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THE BIOCHEMICAL EFFECTS OF INJECTIONS OF SEX HORMONES INTO HYPOPHYSECTOMIZED RATS

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STUDIES OF the biochemical effects of injections of diethylstilbestrol and testosterone propionate into intact (Buchwald and Hudson, 1944) and castrated (Buchwald and Hudson, 1945) rats have been reported. In the intact male rats injected with diethylstilbestrol the acid phosphatase activity of the serum and the alkaline phosphatase activity of the femurs were decreased but no change was effected in the alkaline phosphatase activity of the serum. Testosterone propionate in female rats produced an increase in alkaline phosphatase activity of the serum without influencing the acid phosphatase activity or the alkaline phosphatase activity of the femurs. Diethylstilbestrol and testosterone propionate produced no change in the serum calcium and phosphorus or the output of calcium and phosphorus in the feces in intact males and females, respe tively.

Castration increased the level of acid phosphatase activity of the blood in both male and female rats. As in the intact male rats diethylstilbestrol decreased the acid phosphatase activity of the serum but did not change the alkaline phosphatase activity of the femurs. Diethylstilbestrol did not change the serum calcium and phosphorus or the calcium and phosphorus output in the feces of castrated male rats. The only change produced by testosterone propionate was an increase in alkaline phosphatase activity of the femurs.

The importance of these changes as related to the treatment of carcinoma of the prostate was discussed. Since the prostate is under the influence of the gonadotropic hormone of the hypophysis as well as the testicular hormone, the next step in this investigation was to study the action of the sex hormones in the absence of the hypophy-

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sis. Herger and Sauer (1945) have investigated the inactivation with x-rays of the hypophysis of patients having carcinoma of the prostate. In this study we will report the effects of diethylstilbestrol and testosterone propionate on hypophysectomized rats.

EXPERIMENTAL

An attempt was made to inactivate the hypophysis by means of x-rays but we were unable to cause any retardation in the growth rate of rats by this means. Because massive doses of x-rays produce only a temporary effect on the hypophysis (Lawrence et al., 1937, and Denniston, 1942) this mode of inactivation was abandoned in favor of its surgical removal.

Albino rats of the Wistar strain 100–125 days old were hypophysectomized under ether anaesthesia using the parapharyngeal ap-

TABLE 1. EFFECTS OF INJECTING DIETHYLSTILBESTROL INTO HYPOPHYSECTOMIZED MALE RATS

	1	2	3	4	5
	Noninjected	Oil	Diethylstilbestrol		
Number of rats	12	12	12		
Weight difference, gm.	−7 ±1.66	−5 ±2.25	−38 ± 5.26	0.72	5.77
Femurs, fresh weights, gm.					
1.	0.70±0.013	0.70±0.009	0.79± 0.021	0.00	3.94
2.	0.69±0.015	0.69±0.011	0.79± 0.020	0.00	4.38
Femur, weight of ash, mg.	305.9 ±5.87	305.7 ±5.22	357.0 ±10.81	0.03	4.27
Sex organs, weight, gm.	0.42±0.017	0.44±0.035	0.51± 0.034	0.51	1.43
Serum					
Calcium, mg. %	9.82±0.084†	9.98±0.099*	9.88± 0.069‡	1.23	0.83
Phosphorus, mg. %	5.21±0.086†	5.64±0.122*	5.65± 0.16‡	2.88	0.49
Phosphatase units per 100 cc.					
Acid	15.9 ±1.09	16.0 ±0.64	7.5 ± 0.85	0.08	7.99
Alkaline	106.1 ±6.53	132.9 ±9.00	97.8 ±11.45	2.41	2.41
Bone					
Alkaline phosphatase, units per gm.	4.8 ±0.42	4.1 ±0.34	5.9 ± 0.42	1.30	3.33

* 9 rats.
† 7 rats.
‡ 10 rats.

proach developed by Smith (1930) with some modifications to suit our convenience. Injections were started when these animals were 139–155 days old. The experimental procedure and analytical methods were the same as previously described (Buchwald and Hudson, 1944). All operated rats were placed on the experimental regime and it was not until the end of the experiment when the animals were autopsied that it was definitely established whether the hypophysis had been completely removed. Only the rats in which completeness of ablation was demonstrated at autopsy comprise the hypophysectomized groups. All others are classed as subtotally hypophysectomized.

DISCUSSION

Table 1 gives the results of injecting 0.1 mg. of diethylstilbestrol daily into hypophysectomized male rats. Table 2 gives the results of injecting 0.2 mg. of testosterone propionate daily into hypophysectomized female rats. Columns 1, 2, and 3 show the means with their

probable errors. Column 4 shows the statistical comparison between noninjected and oil injected hypophysectomized rats and Column 5 the comparison between the oil and hormone injected hypophysectomized rats. These values had to be over three to be considered significant. Table 3 gives the results on subtotally hypophysectomized rats. The weight of the sex organs for the males represents the weight of both testicles. For the females the ovaries, oviducts, and uterus were weighed and reported as a unit.

Reported data on serum phosphatase activity, calcium, and phosphorus in hypophysectomized animals are meager. Jones and Shinowara (1942) reported an increase in serum calcium and alkaline phosphatase activity and a decrease in phosphorus. These observations were made on two control and three hypophysectomized female

TABLE 2. EFFECTS OF INJECTING TESTOSTERONE PROPIONATE INTO HYPOPHYSECTOMIZED FEMALE RATS

	1	2	3	4	5
	Noninjected	Oil	Testosterone propionate		
Number of rats	13	12	12		
Weight difference, gm.	2 ±1.44	−3 ±2.39	11 ±3.21	1.79	3.50
Femurs, fresh weights, gm.					
1.	0.57±0.008	0.56±0.011	0.57±0.006	0.74	0.80
2.	0.56±0.008	0.56±0.010	0.57±0.008	0.00	0.78
Femur, weight of ash, mg.	260.4 ±2.94	258.9 ±4.66	265.4 ±3.90	0.27	1.07
Sex organs, weight, gm.	0.19±0.015	0.31±0.020	0.46±0.018	4.80	5.58
Serum					
Calcium, mg. %	10.24±0.093*	9.83±0.142†	9.75±0.117*	2.42	0.43
Phosphorus, mg. %	4.86±0.140*	5.41±0.224†	5.84±0.310*	2.08	1.12
Phosphatase, units per 100 cc.					
Acid	12.9 ±1.09	9.8 ±0.91	8.1 ±0.22	2.18	1.82
Alkaline	112.7 ±4.40	89.6 ±5.29	105.4 ±4.65	3.36	2.24
Bone					
Alkaline phosphatase, units per gm.	4.0 ±0.42	4.7 ±0.48	5.2 ±0.70	1.10	0.59

* 7 rats.
† 6 rats.

rats sacrificed at 153 days of age, 70 days after hypophysectomy. Li et al. (1946) using immature rats, both male and female, found the removal of the pituitary reduced the alkaline phosphatase activity of the plasma. In our investigation serum calcium and phosphorus were lowered in both the hypophysectomized male and female rats as compared with the intact rats (Tables 1 and 2, Buchwald and Hudson, 1944). The acid and alkaline phosphatase activity of the serum of both males and females was increased by hypophysectomy. The increase was greater than that produced by castration (Tables 1 and 2, Buchwald and Hudson, 1945). It might well be that hypophysectomy accentuates the effect produced by castration, since hypophysectomy is the best experimental means of producing atrophy of the sex organs, especially the prostate (Huggins and Russell, 1946). The alkaline phosphatase activity of the bone was depressed by hypophysectomy. The well known effects of hypophysectomy, cessation of growth and atrophy of the sex organs, were evident in our hypophysectomized rats.

In the hypophysectomized male rat diethylstilbestrol produced results similar to those in the intact (Table 1, Buchwald and Hudson, 1944) and castrated (Table 1, Buchwald and Hudson, 1945) rats. Diethylstilbestrol produced a decrease in body weight, without a corresponding decrease in the skeleton weight as judged by the weights of the femurs. Again as in the castrated and intact males, diethylstilbestrol produced a decrease in the acid phosphatase activity while the alkaline phosphatase activity of the serum was not changed significantly. The alkaline phosphatase activity of the bone was

TABLE 3. SUBTOTALLY HYPOPHYSECTOMIZED RATS

	Females Control	Males Control	Males Diethylstilbestrol
Number of rats	10	12	13
Weight difference, gm.	11 ±1.52	21 ±2.43	−44 ±3.23
Femurs, fresh weights, gm.			
1.	0.61 ±0.011	0.82 ±0.016	0.78 ±0.012
2.	0.60 ±0.011	0.82 ±0.014	0.77 ±0.012
Femur, weight of ash, mg.	276.9 ±5.29	367.6 ±7.80	355.3 ±8.19
Sex organs, weight, gm.	0.70 ±0.038	2.71 ±0.040	0.51 ±0.018
Serum			
Calcium, mg. %	10.09 ±0.159*	9.91 ±0.072	10.23 ±0.123
Phosphorus, mg. %	5.24 ±0.218*	6.28 ±0.163	6.09 ±0.127
Phosphatase, units per 100 cc.			
Acid	7.1 ±0.34	8.3 ±0.37	5.6 ±0.26
Alkaline	71.5 ±3.43	82.1 ±4.63	117.3 ±6.77
Bone			
Alkaline phosphatase, units per gm.	6.9 ±0.60	7.2 ±0.71	8.7 ±0.59

* 9 rats.

somewhat increased by diethylstilbestrol, yet the level was about the same as that found in the intact males treated with diethylstilbestrol (5.6 ± 0.34 units per gm.) and lower than that in the castrated males receiving diethylstilbestrol (7.2 ± 0.49 units per gm.).

The effect of testosterone propionate on hypophysectomized female rats was similar to that on castrated rats (Table 2, Buchwald and Hudson, 1945). There was an increase in body weight, although not as great as in the intact (Table 2, Buchwald and Hudson, 1944) and castrated rats. The question of the action of testosterone propionate on the body weight in the absence of the hypophysis has been proposed by Shay et al. (1941). It appears from our observations that the body weight increase at least in part is due to the effect of testosterone propionate per se rather than by way of the hypophysis. Levin (1944) has observed a gain in weight of hypophysectomized rats forcibly fed quantities of food approximately isocaloric with those consumed by normal rats. It is possible that hypophysectomized rats given testosterone propionate and forced to consume sufficient quantities of an adequate diet would show the same weight increase as the intact and castrated rats. Testosterone propionate also produced

an increase in weight of the sex organs in the hypophysectomized as in the castrated females. This increase in weight of the sex organs (ovaries, oviducts, and uterus weighed as a unit) probably reflects an increase in weight of the uterus only since Leathem and Starkey (1939) have demonstrated an increase in weight of the uterus of immature hypophysectomized rats following single massive doses of

TABLE 4. EFFECTS OF THE INJECTION OF SEX HORMONES ON THE CALCIUM AND PHOSPHORUS METABOLISM IN HYPOPHYSECTOMIZED RATS.

	Calcium intake mg. Ca	Calcium output mg. Ca	Calcium balance mg. Ca	Phosphorus intake mg. P	Phosphorus output mg. P	Phosphorus balance Mg. P
			Oil			
			Female 31, Litter 55			
Preliminary, 7 days	918	849	+ 69	730	578	+152
Experimental, 28 days	4015	3965	+ 50	3187	2198	+989
			Female 1, Litter 35			
Preliminary, 7 days	1035	954	+ 81	808	696	+112
Experimental, 28 days	3605	3480	+125	2810	2366	+444
			Testosterone Propionate			
			Female 32, Litter 55			
Preliminary, 7 days	812	724	+ 88	645	509	+136
Experimental, 28 days	3567	3280	+287	2835	1822	+1013
			Female 2, Litter 35			
Preliminary, 7 days	1094	1146	− 52	855	723	+132
Experimental, 28 days	4260	4185	+ 75	3320	2736	+584
			Oil			
			Male 3, Litter 35			
Preliminary, 7 days	797	874	− 77	621	583	+ 38
Experimental, 28 days	3170	2943	+227	2470	2011	+459
			Male 110, Litter 109			
Preliminary, 7 days	696	761	− 65	558	557	+ 1
Experimental, 28 days	3110	3378	+268	2492	2379	+113
			Diethylstilbestrol			
			Male 4, Litter 35			
Preliminary, 7 days	1270	1146	+124	990	733	+253
Experimental, 28 days	4220	3591	+629	3290	2410	+880
			Male 113, Litter 109			
Preliminary, 7 days	750	758	− 8	602	593	+ 9
Experimental, 28 days	1840	1877	− 37	1475	1558	− 83

testosterone propionate while Nathanson et al. (1938) have shown that the hypophysectomized rat ovary is not stimulated by testosterone propionate. The calcium, phosphorus, and acid and alkaline phosphatase activity of the blood serum and the alkaline phosphatase activity of the femurs of hypophysectomized female rats was unchanged by injecting testosterone propionate.

In Table 3 the "control" groups contain for the females the combined data on two injected with oil and eight noninjected and for

the males the combined data on four injected with oil and eight noninjected, since in all previous work as well as in this experiment no significant difference exists between the oil and noninjected groups. These rats in which the hypophysis had been incompletely removed might be considered better controls than nonoperated rats because possible effects of the operative procedure would appear here as well as in the groups in which the hypophysis was completely removed. However, in comparing these subtotally hypophysectomized controls with noninjected intact rats previously reported (Table 3, Buchwald and Hudson, 1945) the only difference is a slight decrease in the serum calcium of both the males and the females of the subtotally hypophysectomized groups. Therefore, the operative procedure itself probably had little or no influence on the action of sex hormones in hypophysectomized rats.

In Table 4 the calcium and phosphorus intake and output in the feces are shown for a preliminary seven-day period and the experimental 28-day period. The per cent of calcium and phosphorus in the feces is high enough that any changes in the true calcium and phosphorus metabolism should be indicated by analysis of the feces only. As in the studies of the intact and castrated animals (Buchwald and Hudson, 1944 and 1945) this experiment was designed as an initial test to indicate the desirability of conducting a complete balance study. The food consumption of the hypophysectomized animals was much lower than for the intact animals. These data demonstrate that diethylstilbestrol and testosterone propionate did not influence the calcium or phosphourus metabolism.

The daily body weights for the animals on the metabolism experiment are shown in Figure 1. They are characteristic of the other animals in the respective groups. The hypophysectomized rats receiving oil showed a nearly constant body weight throughout the whole experiment similar to the castrated animals (Figure 1, Buchwald and Hudson, 1945) and in contrast to the intact animals (Figure 1, Buchwald and Hudson, 1944) which showed a normal growth rate. In general, the hypophysectomized rats receiving the hormone injections had the same type of curve as did the intact and castrated rats, the females receiving testosterone propionate showed a steady increase in body weight while the males receiving diethylstilbestrol showed decreasing body weights.

From these studies it would appear that no beneficial effects could be expected by treating carcinoma of the prostate metastatic to bone by inactivating the hypophysis. This is substantiated by the clinical experiences of Herger and Sauer (1945). Hypophysectomy operates contrary to the present clinical criterion in that it increases the level of acid phosphatase activity in the blood serum. When diethylstilbestrol is administered to hypophysectomized male rats the level of acid phosphatase activity in the blood serum is not

lowered to that obtained in the castrated rats injected with diethylstilbestrol. As a matter of fact neither approaches the level found in intact animals given diethylstilbestrol. In spite of the fact that diethylstilbestrol increased the bone alkaline phosphatase activity in hypophysectomized male rats, it is not at as high a level as that in the castrated males receiving diethylstilbestrol (7.2 ± 0.49 units per gm.). It still appears that the best conditions for healing bone lesions in metastatic carcinoma of the prostate would be the use of diethylstilbestrol in combination with orchidectomy. The increase in acid

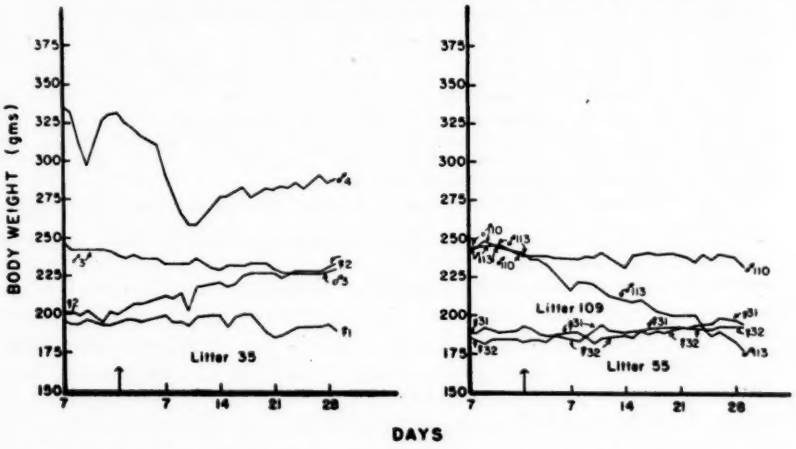

FIG. 1. Daily body weights of hypophysectomized rats on metabolism experiment. Females 1 and 31, males 3 and 110 injected with 0.1 cc. of sesame oil. Females 2 and 32 injected with 0.2 mg. of testosterone propionate in 0.1 cc. of sesame oil. Males 4 and 113 injected with 0.1 mg. of diethylstilbestrol in 0.1 cc. of sesame oil. Arrow indicates day injections were started.

phosphatase activity of the blood serum produced by hypophysectomy appears to be an accentuation of the effect produced by castration.

Herger and Sauer (1943), attempting to explain the delayed failures which occurred in the androgen control of carcinoma of the prostate, suggested that extragonadal stimuli may come from the adrenals. Accordingly, we have initiated experiments to study the effects of sex hormone injections on adrenalectomized rats.

SUMMARY

Daily subcutaneous injections of 0.1 mg. of diethystilbestrol and 0.2 mg. of testosterone propionate were given hypophysectomized male and female rats, respectively, for a 28-day period. There were no changes in the serum calcium, phosphorus, or alkaline phosphatase activity or the output of calcium and phosphorus in the feces.

As in the intact and castrated rats, diethylstilbestrol produced a decrease in the acid phosphatase activity of the blood serum of the males while testosterone propionate in the females had no effect. The level of acid phosphatase activity of the blood serum was increased by hypophysectomy even more than by castration. The level of alkaline phosphatase activity of the bone was decreased by hypophysectomy.

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STUDIES ON INDICES AND CAUSES OF PROLIFICACY IN RATS

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THE objects of this study were twofold: to determine if indices of prolificacy could be developed; to ascertain the cause of the difference in prolificacy in two inbred strains of rats.

Studies with chickens (Hays, 1924), and with turkeys (Asmundson, 1941) have shown an inverse relationship between age at sexual maturity and egg production; the early maturing birds produced a greater number of eggs. Thus it would appear that age at sexual maturity is an index, at least in part, of the fun tional capacity of the ovary in these species. A similar relationship apparently exists between sexual maturity and prolificacy in swine. Phillips and Zeller (1943) found that sexual maturity occurred at an earlier age in large-type Poland China sows than in small-type animals and Hetzer and Brier (1940) working with the same stocks found that prolificacy was greater in the large type. In other words, the more prolific strain matured earlier. In the rabbit, however, Castle (1929) reports that large races mature later than the smaller races and Gregory (1932) found that the large races shed more eggs per ovulation and produced more young.

Can body size be used as an index of prolificacy? Regarding the relationship between species Marshall (191) states, "Broadly speaking, the average number of young produced in a litter in any spe ies of Mammal is inversely proportional to the average size of the animals belonging to that species." He then goes on to state that the sow, a fairly large animal, "is exceptional in having very large litters." The work cited above on swine and the rabbit would indicate a direct relationship between prolificacy and body size within these spe ies. If one compares body size and prolificacy between breeds of swine, however, this same relationship does not maintain. Smith (1937) reports mature weights of Poland China, Duroc Jersey, Yorkshire and Hampshire sows as 795, 766, 609 and 620 pounds respectively. According to data accumulated by Keith (1930) the Poland China has an average litter size of 7.91, the Duroc Jersey 9.93 and the Yorkshire 11.42. Hughes and Ittner (1940) found that the average litter size in 1008 litters from Poland China sows was 7.7, in 1002 litters from Duroc Jersey sows 9.2, and in 302 litters from Hampshire sows 9.2.

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Thus it is obvious that a direct relationship does not invariably exist between prolificacy and body size within a species.

Meager evidence is available to explain variations in prolificacy of animals. It is true that superovulation, superfecundity and increased prolificacy can be produced with the use of gonadotrophins (Engle, 1927; Cole, 1937; Casida et al., 1943; Hammond, 1946; and others) but is the level of gonadotrophin in the blood the factor which *normally* governs prolificacy? Little work has been done on the gonadotrophic level, in the blood of nonpregnant animals because of the difficulties associated with the quantitative extraction of the small amounts of hormone present. The gonadotrophic level in the pituitaries of several species has been studied. Chance, Rowlands and Young (1939) studied the amount of gonadotrophin in the pituitaries of man, horse, pig, sheep and ox and found relative activities, when tested by the use of immature rats, of (man = 100) 100, 30, 1.5, 2.5 and 0.19 respectively. The pig is the most prolific species in the group but its pituitary gonadotrophic titre was next to the lowest.

EXPERIMENTAL

In attempting to produce a more nearly homozygous animal for physiological experimentation, we have developed two strains of rats which have been inbred as the result of sire-daughter or brother-sister matings for 13 or 14 generations. One strain, the grey strain, has an average litter size of 8.9 and the other, the white strain, an average litter size of 6.5. The two strains have a common ancestry as evidenced in that the first 3 sires and the first 3 dams were identical for the two strains. It should be mentioned that no attempt was made to develop strains varying in prolificacy. Breeding stock has been selected on the basis of the performance of the dam. All litters were reduced to 6 at the time of birth. Thus the ability of the dams to raise several litters of 6 young with a good weaning weight received more consideration in selecting young for breeding than the litter size at birth.[1]

INDICES OF PROLIFICACY

Our attention was directed toward the possibility of developing indices of prolificacy by the fact that the weights of ovaries from animals serving as controls for gonadotrophin assays differed markedly for the two strains. The question then arose if the smaller ovaries of the white strain at the 27th day of age might be explained by late sexual maturity. Subsequent investigations showed that both age at sexual maturity and ovarian weight were correlated with prolificacy.

Age at sexual maturity and prolificacy: In determining age at

[1] In studies relating to prolificacy, the diet of the experimental animals is of considerable importance. The one which we used throughout has been found to be adequate for growth and lactation. It consists of: NaCl—8, $CaCO_3$—15, Butter—52, whole milk powder—100, unwashed casein—150, ground wheat—675. A small amount of green alfalfa was fed separately twice weekly.

sexual maturity, the females were examined daily for vaginal opening. Beginning on the day of rupture of the vaginal-closure membrane, vaginal smears were taken and sexual maturity was considered to be reached on the day of the first estrous smear. Our results indicate that age at sexual maturity is correlated with prolificacy, the more prolific strain maturing at an earlier age (table 1). It may be observed that only 3 rats of the white strain matured before the 40th day whereas all but one grey female matured prior to that date. The average age of sexual maturity for the white strain was 41.7 and for the grey strain 36.0 days. The difference between the two means is highly

TABLE 1. AGE AT SEXUAL MATURITY OF GREY AND WHITE STRAINS

Strain	No. of females	Age at sexual maturity of individual females	Ave. age at sexual maturity	Significance of the difference between the means
White	20	47, 45, 45, 44, 44, 43, 42, 42, 42, 42, 42, 41, 41, 40, 40, 40, 40, 39, 38, 38	41.7	$t=6.6$ and $P<0.01$
Grey	25	40, 39, 39, 38, 37, 37, 37, 37, 36, 36, 36, 35, 35, 35, 35, 35, 34, 34, 34, 34, 34, 34, 34, 33, 33	36.0	

significant. The relationship between age at sexual maturity and prolificacy is similar to that found in chickens, turkeys and swine. Thus the possibility exists that age at sexual maturity may serve as an index of the functional capacity of the ovary in certain species and would therefore serve as an index of prolificacy in the rat, barring those conditions in which a normal percentage of eggs shed were not fertilized or carried through normal intrauterine development.

Ovarian weight and prolificacy: Next, let us consider the relationship between ovarian weight and prolificacy, with weight of ovaries determined on the 27th day of age. Both ovaries were dissected free of bursa, oviduct, and supporting tissue, the excess blood removed by blotting on filter paper, and the ovaries weighed on a torsion balance accurate to 0.2 mg.

There is no overlapping in ovarian weights of the two strains, the heaviest pair from the white strain weighing 13.5 mg. and the lightest pair from the grey strain 13.6 mg. (table 2). The means are 10.4 and 17.9 mg. respectively; in other words, the ovaries from rats of the grey strain are 72 per cent heavier than those of the white strain. Thus the possibility of using ovarian size as an index of prolificacy, subject to the same limitations mentioned for age at sexual maturity, is apparent.

It is not only in the immature animal that significant differences in

ovarian weight are found. As we shall show later the ovaries of mature animals likewise differ in weight but to a less marked degree and, as a matter of fact, more nearly in proportion to the difference in prolificacy.

Body weight and prolificacy: The grey strain at 85 to 90 days of age is approximately 10 per cent heavier than the white strain whereas it is 37 per cent more prolific. This represents another instance, therefore in which body size and prolificacy are correlated within a species.

TABLE 2. OVARIAN WEIGHTS AT 27 DAYS OF AGE OF GREY AND WHITE STRAINS

Strain	No. of females	Individual ovarian weights	Ave. ovarian weights	Significance of the difference between the means
White	22	13.5, 13.0, 12.0, 12.0, 11.5, 11.4, 10.6, 10.5, 10.5, 10.4, 10.4, 10.3, 10.2, 10.0, 10.0, 9.7, 9.6, 9.5, 9.2, 8.7, 8.4, 8.2	10.4	$t=12.2$ and $P<0.01$
Grey	26	21.4, 20.5, 20.5, 20.5, 20.0, 19.8, 19.7, 19.3, 19.2, 19.2, 19.0, 18.6, 18.5, 18.2, 18.2, 18.0, 18.0, 17.4, 16.4, 16.0, 15.2, 15.2, 14.4, 14.0, 13.8, 13.6	17.9	

However, as the difference in body weights of the 2 strains is much smaller than the difference in prolificacy, it is doubtful that size could be used as an index of prolificacy.

CAUSE OF THE DIFFERENCE IN PROLIFICACY IN THE 2 STRAINS

General reduced function of the pituitary: One possibility is that all activities of the anterior pituitary are reduced in the low prolificacy strain and that lowered prolificacy is but one evidence of this decreased activity. To test this assumption we have determined the body weight and body length, as well as the weight of the ovaries, thyroids and adrenals of sexually mature females 85 to 90 days of age (table 3).

The pituitaries were also weighed but the excess blood was not removed by blotting because they were to be used for implantation. We felt that the removal of excess blood by blotting, as was done with the other organs, might interfere with the effectiveness of the implants. Consequently the pituitary weights are not shown in the table. The average pituitary weight for the white strain, as determined, was 7.6 mg. as compared to 8.3 mg. for the grey strain. Because of great individual variation, which is probably due in part to differences in amount of excess adhering blood, the differences are not statistically significant.

The white strain is significantly smaller in body size and has smaller ovaries and thyroids even with the weights of these organs expressed in mg. per 100 gm. body weight (table 3). Since all of these factors are controlled to a considerable degree by the anterior pituitary this would speak for a generally reduced function of the anterior pituitary. The white strain has larger adrenals, however; a fact not in accord with this view. Thus, we are unable by this means to obtain clear-cut proof that the reduced prolificacy of the white strain is due to a generally reduced function of the anterior pituitary.

Thyrotropic hormone content of pituitary. As shown in table 3 the thyroids of the white strain expressed in mg. per 100 gm. body weight

TABLE 3. A COMPARISON OF BODY WEIGHT AND LENGTH, OVARIAN, THYROID AND ADRENAL WEIGHTS IN GREY AND WHITE STRAINS. TWENTY-FOUR SEXUALLY MATURE FEMALES 85 TO 90 DAYS OLD OF EACH STRAIN WERE USED

Item compared	White strain	Grey strain	Significance of difference between the two means
Ave. body wt. in gm.	196	216	$t=3.82$ and $P<0.01$
Ave. nose-tail length in cm.	37.4	38.4	$t=3.48$ and $P<0.01$
Ave. ovarian wt. in mg.	63.8	78.1	$t=5.63$ and $P<0.01$
Ave. ovarian wt. expressed in mg. per 100 gm. body wt.	32.5	36.4	$t=3.06$ and $P<0.01$
Ave. thyroid wt. in mg.	19.0	24.4	$t=6.27$ and $P<0.01$
Ave. thyroid wt. expressed in mg. per 100 gm. body wt.	9.71	11.3	$t=4.16$ and $P<0.01$
Ave. adrenal wt. in mg.	40.2	37.6	$t=2.09$ and $P=0.02$
Ave. adrenal wt. expressed in mg. per 100 gm. body wt.	20.7	17.5	$t=5.02$ and $P<0.01$

are smaller than those of the grey strain. An attempt was made to compare the thyrotropic hormone content of the pituitaries of the 2 strains. Eight pituitaries, 2 pituitaries daily for 4 days, were implanted into each day-old chick. A glass canula drawn to a fine point was used in making the pituitary implants in the leg muscle. The canula was attached to a syringe by means of a rubber tube and sufficient sterile saline drawn into the syringe to force the pituitaries out of the canula. The donors were placed under light ether anesthesia, the body length measured and the rat decapitated. The pituitaries were halved and implanted without delay.

Eight chick recipients received implants from grey donors and 9 received implants from white donors. Rats of similar age were compared. The average thyroid weight of the grey rat pituitary recipients was 8.0 as compared to 7.5 mg. for those receiving implants from white donors. Because of considerable individual variation in response the difference is not significant. The data do suggest, however, a greater thyrotropic hormone content in the pituitaries of the grey strain animals.

In spite of our inability to show distinct differences in thyrotropic hormone content of the pituitary, the fact remains that the thyroids

of the grey strain are larger. It is entirely possible that differences in thyroid function of the 2 strains may explain in part the differences in prolificacy.

Gonadotrophin content of the pituitary. We next studied the amount of gonadotrophin in the pituitaries of the 2 strains. Each immature female rat recipient received one pituitary gland daily for 5 consecutive days. The donors were 85 to 90 days of age. Seven recipients receiving pituitaries from white female donors had an average ovarian weight of 61.8 mg. as compared to 57.9 mg. for 8 recipients of pituitaries from grey female donors. Two recipients receiving implants from white male donors had an average ovarian weight of 103.7 mg. as compared to 141.4 mg. for 2 recipients of pituitaries from grey male donors. Neither are the differences statistically significant nor, because of rather wide variation in response, did it appear that increasing the number of recipients within reasonable limits would give a significant difference. Thus, no difference in gonadotrophin content of the pituitaries could be demonstrated by the procedure employed. This, of course, is far from saying that differences do not exist or that a difference in secretory rate does not exist even though the gonadotrophin content of the pituitaries is the same.

Our state of knowledge regarding the relationship between the hormonal content of a gland and its secretory rate is not very complete. As regards the thyroid, there is, under some conditions, no direct correlation between the amount of thyroxin in the gland and the rate of secretion. In hyperthyroidism for example, the thyroxin-like fraction of the thyroid tends to be less than normal (Wilson and Kendall, 1916). After iodine therapy of hyperthyroidism the organically bound iodine increases (Gutman et al., 1932) while the basal metabolism drops (Means and Lerman, 1935).

There is some information on hormonal content of the anterior pituitary and secretory rate. Burrows and Byerly (1936) found more lactogenic hormone in the pituitaries of broody than in laying hens. Broodiness is presumably brought about by increased lactogenic hormone secretion and, therefore, in this instance a high hormonal content of the gland is apparently correlated with a high secretory rate. Reece and Turner (1937) studied the lactogenic hormone content of lactating and dry beef and dairy cows. Assuming that the rate of milk secretion depends upon the rate of lactogenic hormone secretion one would expect the pituitaries of dairy cows to contain more hormone than beef cows and the pituitaries of lactating animals more than those of dry cows. Their data faintly suggest that this is the case.

Chen and van Dyke (1936) studied the thyrotropic hormone content of the pituitaries of normal and thyroidectomized female rabbits and found more hormone in the pituitaries of the operated animals. As more thyrotropic hormone is usually found in the blood under conditions in which the thyroxin secretion is low (Hertz and Oastler,

1936), it is probable that the blood of the operated animals was likewise richer in the hormone.

Witschi and Riley (1940) found that the gonadotrophin content of the pituitaries of castrate and menopausal women was high and, as is well known, the secretory rate is high in such individuals. By parabiotic experiments, Kallas (1929) showed that castrated rats secreted more gonadotrophin than normal animals; furthermore, the pituitaries of the castrates were richer in gonadotrophin.

Studies available on the pituitary, therefore, indicate rather uniformly a direct relationship between hormonal content of the gland and secretory rate. Had our findings shown that the pituitaries of the prolific strain contained more thyrotropic hormone or more gonadotrophin than the less prolific strain, the results would have been very suggestive of a higher secretory rate. With the results obtained, however, one can only conclude that, if the increased prolificacy of the grey strain depends upon an increased secretion of gonadotrophin or thyrotropic hormone, the difference in secretory rate is not clearly indicated by the hormonal content of the gland.

SUMMARY

Studies were made on indices of prolificacy and on the cause of the difference in prolificacy of two inbred strains of rats. The less prolific strain, the white strain, had an average litter size of 6.3 as compared to 8.9 for the more prolific strain, the grey strain.

Age at sexual maturity was found to be correlated with prolificacy, the earlier maturing strain having the larger litter size. Twenty-five females of the grey strain matured at an average age of 36.0 days as compared to 41.7 days for 20 females of the white strain.

The more prolific strain had larger ovaries. The average ovarian weight of 26 grey females at 27 days of age was 17.9 as compared to 10.4 mg. for 22 white females.

As regards the cause of the difference in prolificacy, the fact that the body size, ovarian and thyroid weights were significantly less in the white strain (24 sexually mature females of each strain were compared) points toward a general reduction in activity of the anterior pituitary. In opposition to this view, however, the adrenals of the white strain were larger than those of the grey strain. Furthermore, we did not obtain evidence that the pituitaries of grey strain rats contained more thyrotropic hormone (as determined by the weight response of the thyroids in day-old chicks to pituitary implants) or more gonadotrophin (as determined by the ovarian weight response in immature rats to pituitary implants) than the pituitaries of the white strain.

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EFFECT OF ADRENALECTOMY ON THE MAMMARY GLAND OF THE CASTRATED AND ESTROGEN TREATED CASTRATED MALE RAT[1]

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ALTHOUGH the estrogens were early believed capable of stimulating only duct growth of the mammary gland, it became apparent, with the availability of crystalline preparations and the use of larger dosages and longer periods of treatment, that administered estrogens were also capable of producing varying amounts of alveolar development. Certain species apparently respond to estrogen with fuller alveolar development than others, the guinea pig being one of the most responsive (Turner et al., 1934; Lyons et al., 1936; Nelson 1937, and Lewis and Turner, 1942). However, the rate and extent of mammary gland development with estrogen is in general much inferior to that which normally occurs during pregnancy.

Whether or not the dosage of estrogen is within the "physiologic range" is also an important consideration in the interpretation of alveolar mammary responses to estrogens. It has been found in this laboratory that the level of dimethyl ether of diethylstilbestrol (administered in the feed) required to produce alveolar development in male mice over a period of from 3 to 9 weeks also brings about marked retardation of growth or body weight loss (unpublished). The dosages of estrogen generally employed in mammary gland studies are usually sufficient to produce a marked body weight loss.

In the isolated post-pubertal virgin female rabbit maintained on a high nutritional level, a more or less continuous state of estrus exists, uninterrupted by spontaneous ovulation. It is significant that in such animals, under a continuous "physiological" estrogen stimulation, the mammary glands consist of an extensive duct system with very little alveolar development (Turner and Frank, 1930; Turner and Gardner, 1931).

The considerable synergistic effect of corpus luteum extract or progesterone with estrogen upon alveolar development has long been appreciated (Turner and Frank, 1931, 1932; Lyons et al., 1941; Scharf et al., 1941) and the rate and degree of development produced

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by these two hormones combined much more nearly approaches that of pregnancy. Various androgens and desoxycorticosterone have been found to produce effects similar to progesterone in promoting mammary alveolar development (Reece et al., 1939; Speert, 1940; Mixner et al., 1943; Nelson et al., 1943). Since administered estrogen causes adrenal stimulation (Selye et al., 1935, 1936), and since androgens, progesterone, and desoxycorticosterone have been isolated from the adrenal, the question arises as to whether the alveolar response to administered estrogen may be mediated by way of the adrenal (Petersen, 1942). The present experiment was undertaken to determine the relative effectiveness of estrogen on the mammary gland of castrated and castrated-adrenalectomized male rats.

METHODS

Male Wistar rats, from a colony maintained at the University of Missouri for many years, were used. Body weights varied from about 200 to 300 grams, with most of them within the range of 220 to 260 grams. Adrenalectomy was performed under ether anesthesia through a single dorsal skin incision. Adrenalectomized animals were maintained on 1% sodium chloride drinking water. Some strains of rats survive adrenalectomy much better than others, apparently as a result of accessory adrenal tissue. The suitability of this strain for adrenalectomy experiments was determined by the time of survival of adrenalectomized animals following substitution of distilled water for the 1% saline drinking water (Group 1). Castration, where performed, occurred from 41 to 103 days before the animals were used. Adrenalectomy was performed 10 days prior to sacrificing. Estrogen treatment consisted of 5 micrograms daily of α-estradiol benzoate[2] in oil for 10 days prior to sacrificing. In the castrated-adrenalectomized-estrogen treated animals, estrogen treatment was instituted on the day adrenalectomy was performed. Upon sacrificing the adrenalectomized animals, loose connective tissue plus any suspicious nodules were removed from the kidney area of all animals. This was serially sectioned by the paraffin method, stained and examined microscopically for the possible presence of accessory adrenal tissue.

The skins, with attached mammary glands were fixed and stained. All of the mammary glands on each animal were examined and a typical gland removed. This was usually the second or third thoracic gland although in some cases where the two were closely associated they were removed as a unit. Mammary glands were rated on the basis of structure only, gland area not being considered because of the greater variation from one animal to another, and because of the relatively short period of treatment. Mammary glands were mounted for comparison from 4 normal (Group 2), 4 castrate (Group 3), 4 castrated-adrenalectomized (Group 4), 4 castrated-estrogen treated (Group 5), and 12 castrated-adrenalectomized-estrogen treated (Group 6) animals.

[2] The α-estradiol benzoate used in this work was supplied through the courtesy of Schering Corporation, Bloomfield, N. J.

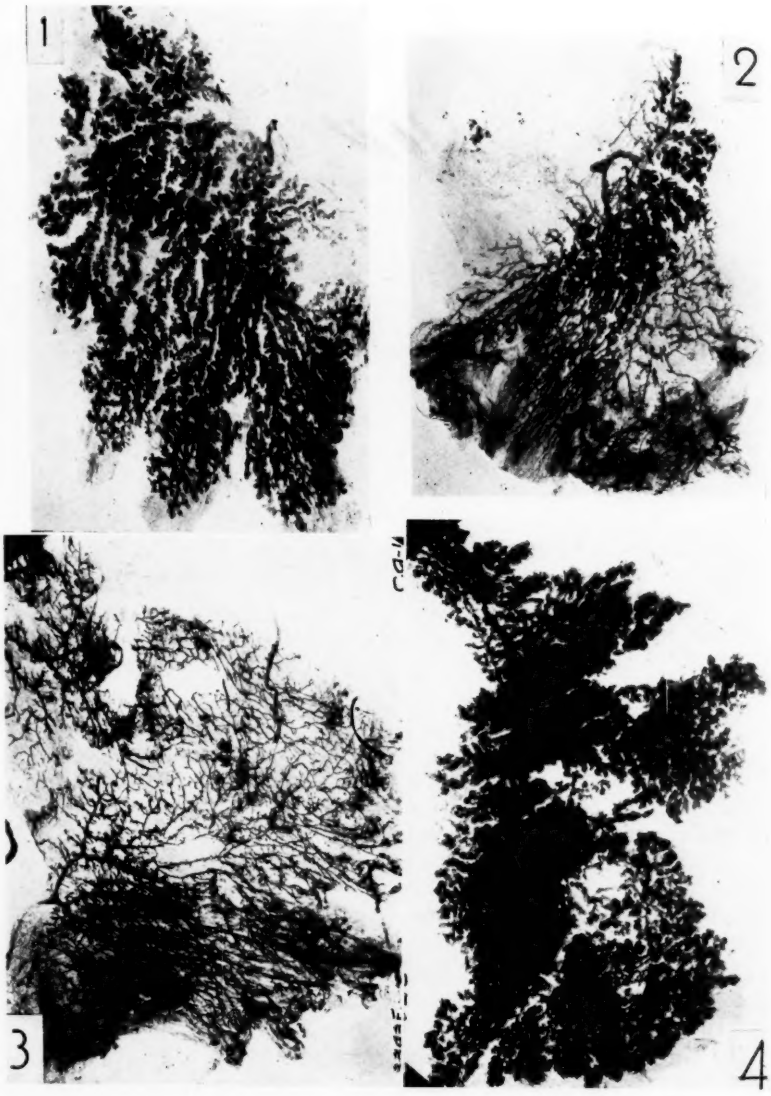

FIG. 1. Mammary gland of normal male rat. 3×
FIG. 2. Mammary gland of male rat castrated 62 days previously. 3×
FIG. 3. Mammary gland of castrated male rat adrenalectomized for 10 days. 3×
FIG. 4. Mammary gland of castrated male rat treated with 5 micrograms of α-estradiol benzoate daily for 10 days. 3×

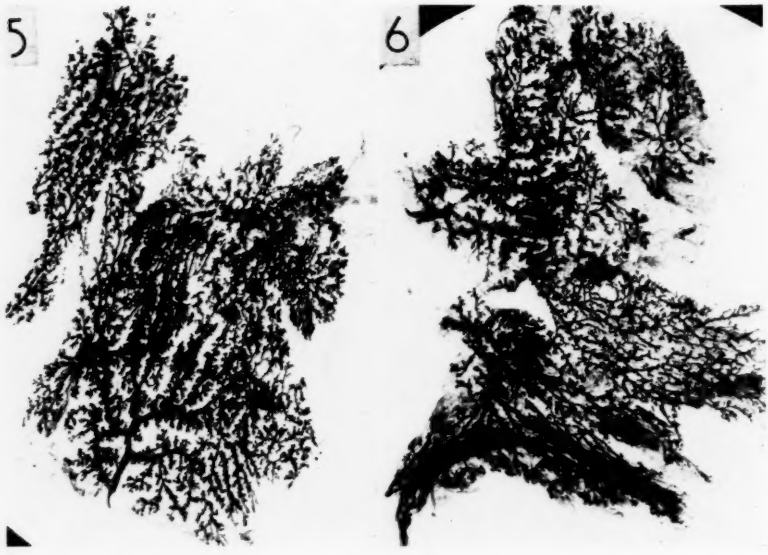

FIG. 5. Mammary gland of castrated male rat adrenalectomized and treated with 5 micrograms of α-estradiol benzoate daily for 10 days. Serial sections revealed a small accessory adrenal node in this animal. 3×

FIG. 6. Mammary gland of castrated male rat adrenalectomized and treated with 5 micrograms of α-estradiol benzoate daily for 10 days. No accessory adrenal tissue was found in this animal. 3×

RESULTS

Of eleven rats (Group 1) adrenalectomized and maintained on 1% saline for 10 days, one died on the 8th day. After substitution of distilled water for the saline on the 10th day, the average survival time of the remaining 10 rats was 14.3 days.

A representative gland from one of the normal male rats is shown in Figure 1. This is typical of the extensive alveolar proliferation found in adult male rats of this strain (Turner and Schultze, 1931).

Glands from the castrated animals were uniformly less extensively developed than the normal. However, the central area of the gland still contained an appreciable amount of alveolar tissue, with the extremities consisting of bare ducts (Figure 2).

In the castrated rats adrenalectomized for 10 days the glands were very atrophic, alveolar development being entirely absent and the ducts very thin (Figure 3).

The castrated estrogen treated animals showed extensive alveolar response, even though they lost an average of 20.6 grams as a result of the 10 days of estrogen treatment (Figure 4).

The mammary gland response of the castrated-adrenalectomized animals to estrogen treatment was strikingly inferior to that of the

castrated animals to the same dose of estrogen. In no case were the mammary glands as atrophic, however, as those of the castrated-adrenalectomized animals without estrogen treatment.

The mammary glands of the castrated-adrenalectomized-estrogen treated animals (Group 6) were rated against the mammary glands of the castrated male rats, in which condition they began the 10 day period of adrenalectomy and estrogen treatment. This comparison was made both on the basis of duct stimulation and alveolar stimulation.

Eleven of the twelve animals (Group 6) showed a stimulation of the periphery of the ducts with end-bud formation. In the twelfth animal the condition of the gland was unchanged as compared to the gland of castrated animals. On the basis of alveolar development, however, 4 animals showed less, 6 animals showed essentially the same, and 2 animals showed more alveolar development than the glands from castrated animals. One of the two animals showing more alveolar development than the castrated controls was the only animal in which accessory adrenal tissue was found in the serial sections. This animal showed the best response of any of the castrated-adrenalectomized animals to estrogen (Figure 5). A more typical gland of this group is shown in Figure 6.

DISCUSSION

The complete mortality of the adrenalectomized animals of Group 1 within a reasonably short time after substitution of distilled water for the saline drinking water indicates the suitability of this strain of rats for adrenalectomy studies. This is in conformity with previous adrenalectomy studies performed on this strain of rats (Meites et al., 1942).

The presence of alveolar tissue in castrated male rats is interesting. That the mammary gland of the adult male rat possesses considerable alveolar development has long been recognized (Turner and Schultze, 1931; Astwood et al., 1937). However, reports as to the effect of castration in the male rat are somewhat conflicting. Turner and Schultze (1931) compared castrated male rats (Wistar) with normal males at monthly intervals and concluded that castration neither inhibits nor hastens the characteristic lobule proliferation. Continued duct growth was also observed. It was suggested that the hormone or hormones causing the marked lobule proliferation of the mature male rat might be produced by some organ other than the gonad.

McEuen, Selye and Collip (1936) using biopsies rather than whole mounts of the mammary glands, reported no secretion or development in 48 and 56 day old male rats (strain not mentioned) that had been castrated at 34 days of age. Biopsies taken 15 and 50 days after castration of 6 males at 4½ months were reported to show marked signs of involution.

Astwood, Geschickter and Rausch (1937) reported that growth of the mammary glands of the male and female rat (albinos obtained commercially) during the first 6 weeks of life proceeds with equal rapidity and is entirely independent of the gonad. In males castrated at 3 weeks of age, mammary growth proceeded until 8 weeks of age, after which it remained unchanged until 16 weeks of age, at which time signs of involution were noted.

Smithcors and Leonard (1942) castrated male rats (Sprague-Dawley strain) at 24 days, and after 41 days all of the mammary glands showed good extension of the duct system but no evidence of aveolar development.

Lewis, Gomez and Turner (1942) reported slight or no regression two weeks after castration with lobule development present in some of the castrate males (albinos, strain not mentioned, probably Wistar).

More recently Cowie and Folley (1947) have observed that in male or female rats (hooded Norway) gonadectomized at weaning, alveolar tissue, although absent in animals killed at 28 days of age, was present in the glands of animals killed at greater ages.

Strain differences undoubtedly account for the variable results. The complete disappearance of alveolar tissue from the glands of castrated male rats within 10 days following adrenalectomy (Figures 2 and 3) indicates the adrenal as the likely source of stimulation in those strains in which the mammary gland grows or is maintained in the absence of the gonad.

In certain strains of mice it has been demonstrated (Woolley *et al.*, 1941; Fekete *et al.*, 1941; Gardner, 1941), that animals of either sex if castrated up to 6 months of age will develop, after 8 or 10 months, adrenal hyperplasia followed by mammary gland growth and signs of estrogenic stimulation of the uterus and vagina.

The present experiments obviously do not bear out previous reports that adrenalectomy in itself enhances mammary growth of rats (Butcher, 1939; Reeder et al., 1944). Quite the opposite effect was obtained. Butcher's work involved underfed animals and is not strictly comparable. Reeder and Leonard obtained their effect only if the animals gained appreciably in weight following adrenalectomy. They point out that this variation might be attributed to variable amounts of functional accessory cortical tissue which was known to be present. Cowie and Folley (1944) report no change in mammary gland structure but a decrease in gland area following adrenalectomy in gonadectomized rats. Cramer and Horning (1939) report mammary involution in adrenalectomized mice. In their recent report Cowie and Folley (1947) observed evidence for slight mammary gland regression after adrenalectomy in two experiments but not in a third.

Comparison of the results of Groups 3, 5, and 6 indicates that although estrogen maintains its ability to stimulate duct growth in

the adrenalectomized rat, its ability to stimulate alveolar development is absent, or at best very greatly diminished. Cramer and Horning (1939) have previously reported that the mammary glands of adrenalectomized mice injected with estrogen never showed such an advanced degree of development and secretory activity as in the similarly treated intact animals. Selye and Masson (1939) on the other hand have reported that the estrogenic effect of estrone and diethylstilbestrol on the vagina and uterus of the rat is not prevented by adrenalectomy.

A restricted food intake has been reported to cause a lowered responsiveness of the mammary gland to estrogen (Astwood et al., 1937; Trentin et al., 1941). Food consumption data were not taken in the present study. However, Cowie and Folley (1947) obtained similar mammogenic effects upon administration of anterior pituitary extract to castrated or castrated-adrenalectomized rats. They reported that while the response may have suffered some diminution in the adrenalectomized rats, the degree of such diminution was relatively slight. It is doubtful, therefore, that the wide divergence in the response to estrogen observed in the present experiment could be attributed entirely to a voluntary decrease in food consumption by the adrenalectomized animals.

It is possible that part of the diminished mammary effect of estrogen in the adrenalectomized rats may be attributed to a toxic effect of the estrogens in adrenalectomized animals. Such an effect has often been reported. In the present work, mortality among the adrenalectomized animals of Group 6 after treatment with estrogen was 1 out of 13. Although this was not higher than in untreated adrenalectomized rats, the 12 remaining rats lost an average of 35.2 grams as compared with an average loss of 18.7 grams by the untreated adrenalectomized animals of Group 5. It will be noted, however, that in the castrated rats with intact adrenals estrogen produced excellent alveolar growth even though the animals lost an average of 20.6 grams as a result of treatment. It would appear probable therefore that the extensive alveolar development produced by administered estrogen may be mediated by way of the adrenals.

SUMMARY

1. In the strain of rats used, castration resulted in a reduction but not complete elimination of the alveolar development normally found in the mammary glands of adult males.

2. Adrenalectomy of castrated male rats and maintenance on 1% saline for 10 days resulted in a complete elimination of the alveolar development, and thin atrophic ducts.

3. Administered estrogen, although causing noticeable duct stimulation, was relatively ineffective in stimulating mammary alveolar growth in adrenalectomized, castrated rats maintained on salt, as compared to castrate rats.

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THE ADRENAL CHOLESTEROL AND ASCORBIC ACID CONTENTS AFTER INJURY*

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SELYE (1946) and Long (1946) have reviewed the responses of the organism to various types of stress. The role of the anterior pituitary and the adrenal hormones in the adaptation phenomena are generally accepted. Decreases in the cholesterol and ascorbic acid content of the adrenals after (1) injection of adrenotrophic hormone (Sayers, Sayers, Fry, White and Long, 1944), (2) hemorrhage (Sayers, Sayers, Liang and Long, 1944), (3) scalding (Harkins and Long, 1945) (Harkins, 1945) and (4) exposure to cold (Levin, 1945) (Long, 1946) are characteristic changes noted a few hours after injury. A decrease in adrenal cholesterol content and concentration occurs a few days after trauma (Popjack, 1944) and after injection of β-chloroethyl vesicants (Ludewig and Chanutin, 1946). The latter investigators pointed out that the decrease in ester cholesterol is responsible for the drop in total cholesterol concentration.

The present experiments were carried out to determine the changes in adrenal cholesterol and ascorbic acid after a variety of stresses over a period of several days. It was found that injury may produce initial decreases but always causes delayed increases in the cholesterol and ascorbic acid contents of the adrenals.

METHODS

Male rats of Wistar stock, about 70 days old and weighing between 150–200 gm., were used as experimental animals. Details have been presented for the intravenous injections of the mustards and saline in rats (Ludewig and Chanutin, 1946). The following agents, symbols and dosages were used.

bis β-chloroethyl) sulfide	(H); 0.4 mg./kg.
ethyl bis (β-chloroethyl) amine HCl	(HN1); 0.4 mg./kg.
methyl bis (β-chloroethyl) amine HCl	(HN2); 0.6 mg./kg.
tris (β-chloroethyl) amine HCl	(HN3); 0.6 mg./kg.

All rats were starved after injections.

H was applied to a 10 cm^2 area of the clipped back of animals anesthetized with sodium pentobarbital. A dosage of approximately 20 mg./kg. was administered by using about 0.2 ml. of a 2.0% petroleum ether solution of H. Each animal was kept under anesthesia in an individual cage in a well

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* This work was done under contract with the Medical Division of the Chemical Corps.

ventilated hood for about 2 hours. At this time the excess H was removed with soap and water. Food was available to these animals during the experimental period.

Thermal injury was produced by dipping the clipped backs of rats anesthetized with sodium pentobarbital in water at 75° for 20, 30 or 60 seconds. Approximately one-third of the body surface was scalded. Rats anesthetized, but not burned, served as controls. Food was always available.

The removal of the adrenals, the analytical procedures for cholesterol determinations and the statistical treatment of the data have been presented in a previous paper (Ludewig and Chanutin, 1946). Ascorbic acid was determined on homogenized trichloroacetic acid extracts by the procedure developed by Roe and Kuether (1943).

Blood was withdrawn from the abdominal aorta by needle and syringe

TABLE 1. ADRENAL WEIGHT AND CHOLESTEROL CONTENT DURING THE FIRST 24 HOURS AFTER INJECTION OF HN3

Hours after injection	Adrenal		
	Weight	Cholesterol	
		Ester	Free
	mg./100 gm. body weight		
	Control, saline injected		
0	11.3 ± .24 (48)‡	.26 ± .01	.038 ± .002*
3	12.3 ± .32 (12)	.19 ± .02*	.030 ± .001*
6	12.5 ± .24*(10)	.17 ± .01*	.031 ± .001
12	14.3 ± .52*(6)	.31 ± .05	.038 ± .004
24	14.8 ± .44*(10)	.29 ± .03	.033 ± .002
	HN3		
3	13.0 ± .19 (11)	.13 ± .01†	.027 ± .002
6	13.2 ± .45 (12)	.16 ± .02	.028 ± .002
12	14.2 ± .39 (6)	.14 ± .01†	.038 ± .002
24	15.6 ± .51 (9)	.18 ± .02	.037 ± .003

* Represents significant difference from 0 day control value.
† Represents significant difference from value of corresponding control group.
‡ Figures in parenthesis represent number of animals.

and prevented from clotting with heparin. One ml. samples of plasma were extracted with an alcohol:acetone (1:1) mixture and analyzed for cholesterol by the method described by Sperry and Brand (1943).

RESULTS

ADRENAL WEIGHT AND CHOLESTEROL. *Effect of HN3 during first 24 hours.* The adrenal weights per 100 gm. rat and the contents of bound and free cholesterol per 100 gm. rat are shown in Table 1. Hypertrophy of the adrenals is noted in both the saline and HN3 rats 3 hours after injection. The adrenals of both groups continue to hypertrophy to about the same extent during the first day.

The cholesterol ester content of the saline controls decreases significantly at the 3rd and 6th hours and returns to the normal range at the 12th hour. After HN3, the values for this constituent decrease significantly from those of the respective saline controls at

the 3rd and 12th hours. The values for the free cholesterol contents of the saline and HN3 groups are almost identical.

Effect of thermal injury. Data are presented in Table 2 for the adrenal weights and for cholesterol of control and burned rats.

TABLE 2. ADRENAL WEIGHT AND CHOLESTEROL CONTENT AFTER THERMAL INJURY

After injury	Weight	Cholesterol	
		Ester	Free
	mg./100 gm. body weight		
	Controls		
0 hours	11.3 ± .24 (48)‡	.26 ± .01	.038 ± .002
3 hours	13.0 ± .52* (9)	.18 ± .01*	.029 ± .003
6 hours	12.1 ± .23 (10)	.19 ± .02*	.031 ± .002
12 hours	13.9 ± .58* (9)	.27 ± .03	.031 ± .003
24 hours	11.3 ± .26 (10)	.23 ± .02	.031 ± .001
	Rats, burned at 75° for 60 seconds		
3 hours	13.2 ± .41 (12)	.16 ± .02	.037 ± .002
6 hours	12.3 ± .27 (12)	.09 ± .01†	.028 ± .001
12 hours	13.0 ± .55 (10)	.07 ± .01†	.031 ± .003
24 hours	15.3 ± .55† (9)	.13 ± .01†	.031 ± .003
2 days	17.2 ± 1.98* (9)	.25 ± .04	.047 ± .004
3 days	14.1 ± .44* (10)	.49 ± .03*	.040 ± .002
4 days	13.6 ± 1.10* (7)	.43 ± .09*	.033 ± .003
5 days	14.6 ± .44* (9)	.38 ± .03*	.041 ± .002
7 days	16.6 ± .89* (8)	.34 ± .04*	.050 ± .003*
	Rats, burned at 75° for 30 seconds		
1 day	15.6 ± .68* (8)	.20 ± .02	.040 ± .003
2 days	13.3 ± .47* (9)	.47 ± .03*	.036 ± .005
3 days	13.5 ± .59* (8)	.46 ± .03*	.036 ± .002
4 days	14.3 ± .54* (9)	.43 ± .03*	.040 ± .002
5 days	14.1 ± .83* (10)	.52 ± .04*	.057 ± .003*
6 days	13.9 ± .47* (9)	.34 ± .02*	.062 ± .002*
	Rats, burned at 75° for 20 seconds		
1 day	13.6 ± .33* (8)	.28 ± .02	.036 ± .003
2 days	13.5 ± .45* (9)	.41 ± .03*	.042 ± .003
3 days	12.1 ± .31* (8)	.39 ± .04*	.040 ± .002
4 days	13.2 ± .58* (9)	.33 ± .01*	.045 ± .003
5 days	13.2 ± .53* (10)	.40 ± .02*	.058 ± .003*
6 days	14.0 ± .47* (10)	.47 ± .02*	.073 ± .003*

* Represents significant difference from 0 day control value.
† Represents significant difference from value of corresponding control group.
‡ Figures in parenthesis represent number of animals.

A brief period of anesthesia in normal rats is responsible for a significant increase in the adrenal weight/100 gm. rat at the 3rd and 12th hours with a return to the original weight at the 24th hour. A similar increase is seen for rats severely burned for 60 seconds during the first 12 hours. All the adrenal weights are significantly greater than those of the normal controls from the 1st to the 7th days after scalding.

The anesthesia produced by sodium pentobarbital is responsible for significant decreases in the ester cholesterol content at the 3rd and 6th hours. The values return to the normal range at the 12th hour.

After burning for 60 seconds the bound cholesterol values are depressed below those of the controls during the first 24 hours. During this same period, the free cholesterol content is not influenced by this injury. All but one of the mean values for the ester cholesterol contents increase significantly after the first day over the control regardless of the degree of thermal injury. The free cholesterol content increases somewhat during the 5th, 6th or 7th days after burning.

Effect of cutaneous application of H (Table 3). Comparatively large doses of sulfur mustard cause a hypertrophy of the adrenals. A significant increase in the bound cholesterol is first noted on the 4th day after H. These values remain high for the experimental period. The free cholesterol content of the adrenals does not vary appreciably in these animals.

TABLE 3. ADRENAL WEIGHT AND CHOLESTEROL CONTENT AND TOTAL AND FREE PLASMA CHOLESTEROL AFTER CUTANEOUS APPLICATION OF H

Days after application	Adrenal			Plasma cholesterol	
	Weight	Cholesterol		Total	Free
		Ester	Free		
	mg./100 gm. body weight and standard error			mg./100 ml.	%
0	11.3 ± .24 (48)†	.26 ± .01	.038 ± .002	61 ± 2.6 (10)	25 ± .98
1	14.6 ± .34*(10)	.23 ± .02	.038 ± .015	59 ± 2.8 (10)	29 ± 1.2
2	16.0 ± .98*(10)	.27 ± .04	.045 ± .004	63 ± 3.2 (9)	30 ± 1.4
3	15.9 ± .89*(10)	.30 ± .04	.042 ± .001	65 ± 2.8 (10)	26 ± 1.8
4	17.9 ± 1.19*(10)	.42 ± .03*	.046 ± .002	60 ± 4.7 (10)	31 ± 1.4*
5	20.5 ± 2.69*(9)	.45 ± .06*	.053 ± .006	42 ± 2.1*(9)	30 ± 3.0
6	17.2 ± 2.19*(8)	.44 ± .05*	.042 ± .004	48 ± 3.3*(8)	28 ± 2.9
7	15.7 ± .78*(8)	.40 ± .04*	.039 ± .002		

* Represents significant difference from 0 day control value.
† Figures in parenthesis represent number of animals.

PLASMA CHOLESTEROL. *Effect of cutaneous application of H.* These results are shown in Table 3. The total cholesterol concentrations remain constant for 4 days and then decrease significantly on the fifth and sixth days. The mean values for the percentage of the free cholesterol of the H treated animals remain constant.

In a previous study it was also shown that the plasma total cholesterol concentration of the rat is not appreciably affected by injury (Chanutin and Ludewig, 1947). No relationships could be established between the changes in cholesterol observed in the adrenals and the plasma.

ADRENAL ASCORBIC ACID. *Effect of mustards.* The data for ascorbic acid are presented in terms of content per 100 gm. rat and as percentage concentration (Table 4). In the saline controls there is a marked decrease in the ascorbic acid content and concentration at the 3rd hour. The return of the ascorbic acid content to the normal range and the continued decrease in percentage concentration during the 6th

and 12th hours are explained by the hypertrophy of the adrenals. The values for the ascorbic acid content are above normal at the end of the first day and continue to increase.

Comparisons of the mean values of the HN3 injected rats with the respective saline control values show no differences during the first 24 hours. The values are increased on the 2nd and 3rd days for the HN1,

TABLE 4. ADRENAL ASCORBIC ACID AFTER INJECTION OF HN1, HN2, HN3 AND H

After injection	Saline	HN3	HN1	HN2	H
		mg./100 gm. body weight			
0 Hours	.054 ± .002 (10)‡				
3 Hours	.039 ± .004* (6)	.038 ± .003 (16)			
6 Hours	.050 ± .002 (6)	.044 ± .002 (6)			
12 Hours	.053 ± .002 (10)	.051 ± .002 (10)			
24 Hours	.068 ± .003* (17)	.077 ± .005 (20)	.075 ± .002 (11)	.072 ± .002 (10)	.066 ± .003 (10)
2 Days	.067 ± .002* (17)	.082 ± .007 (12)	.084 ± .006† (8)	.093 ± .003† (12)	.087 ± .004† (10)
3 Days	.081 ± .003* (18)	.099 ± .004* (12)	.095 ± .003† (11)	.097 ± .001† (9)	.096 ± .003† (10)
		mg./100 gm. adrenal tissue			
0 Hours	.49 ± .02				
3 Hours	.28 ± .01*	.29 ± .02			
6 Hours	.37 ± .01*	.33 ± .02			
12 Hours	.38 ± .01*	.35 ± .01			
24 Hours	.43 ± .01	.46 ± .01	.42 ± .02	.42 ± .01	.42 ± .02
2 Days	.45 ± .01	.46 ± .02	.44 ± .03	.47 ± .01	.49 ± .02
3 Days	.45 ± .01	.46 ± .02	.48 ± .02	.45 ± .02	.46 ± .01
4 Days	.48 ± .02				
5 Days	.48 ± .02				

* Represents significant difference from 0 day control value.
† Represents significant difference from value of corresponding control group.
‡ Figures in parenthesis represent number of animals.

TABLE 5. ADRENAL ASCORBIC ACID AFTER SCALDING WITH HOT WATER (75°) FOR 60 SECONDS

Hours after injury	mg./100 gm. body weight		mg./100 mg. adrenal tissue	
	Control	Burn	Control	Burn
0	.054 ± .002 (10)‡		.49 ± .02	
3	.042 ± .002*(9)	.029 ± .001†(10)	.38 ± .01*	.24 ± .01†
6	.054 ± .002 (9)	.035 ± .002†(10)	.43 ± .02	.28 ± .01†
12	.075 ± .003*(10)	.042 ± .002†(9)	.51 ± .01	.33 ± .01†
24	.065 ± .004 (9)	.059 ± .002 (10)	.51 ± .02	.39 ± .01†
48		.067 ± .004*(10)		.44 ± .02
72		.070 ± .004*(10)		.46 ± .02

* Represents significant difference from 0 day control value.
† Represents significant difference from value of corresponding control group.
‡ Figures in parenthesis represent number of animals.

HN2 and H groups and on the 3rd day for the HN3 groups. The values for the percentage concentrations do not vary from those of the respective controls.

Effect of thermal injury (Table 5). Sodium pentobarbital anesthesia alone causes a decrease at the 3rd hour and an increase above normal at the 12th hour but these variations of the ascorbic acid content are slight. After burning, the ascorbic acid content is markedly decreased immediately and the values remain low during the first 12 hours; the ascorbic acid contents return to the normal range

at the end of the first day. The results for the percentage concentrations are similar to those described for the contents.

DISCUSSION

In a previous paper (Ludewig and Chanutin, 1946), hypertrophy of the adrenals was noted on the 1st day after starvation or after the injection of β chloroethyl vesicants. In the present investigation, this hypertrophy is apparent as early as the 3rd hour after; (a) anesthesia, (b) anesthesia plus saline injection, (c) severe burns, and (d) intravenous injection of HN3. The adrenal weights return to the control range within 24 hours only in the anesthetized rats. These data illustrate the rapidity of the response of adrenals to minimal injury.

The most marked adrenal hypertrophy in the series of experiments is noted in the rats treated cutaneously with sulfur mustard. The skin lesions in these animals were not particularly severe; diarrhea was noted in about half the animals treated. In the scalded animals, the extent of hypertrophy is related to the time of exposure to hot water.

The influence of various stresses on the cholesterol and ascorbic acid concentrations of the adrenals is discussed by Long (1947). According to this investigation, Nembutal anesthesia causes little change in the cholesterol and ascorbic acid concentrations within the first $2\frac{1}{2}$ hours. In the present experiments, approximately 50 mg. per kg. of Nembutal induces anesthesia in rats for 45-60 minutes. In these animals the cholesterol content and concentration is decreased significantly on the third and sixth hours. The ascorbic acid content is decreased slightly at the third hour. Similar changes for cholesterol and ascorbic acid are also noted after intravenous saline injections into anesthetized animals. These results indicate that the evaluation of adrenal cholesterol and ascorbic acid changes, especially during the first 12 hours following stress, must depend on adequate control data.

In order to demonstrate more clearly the effects of vesicants and scalding on the bound cholesterol and ascorbic acid contents of the adrenals, the percentage changes from the control values are shown in Figures I and II.

After injection of HN3 there are initial decreases in cholesterol content during the first 24 hours. After HN3 and H injection the greatest decreases are observed on the 3rd and 4th day respectively (Ludewig and Chanutin, 1946). Precipitous increases in cholesterol content are noted after this time. Such increases are difficult to interpret in dying animals. After cutaneous application of H there is a pronounced increase in the ester cholesterol content. The effects of administering lethal doses of H by two different routes probably represent a difference in the mechanism responsible for death.

The adrenal cholesterol content of rats treated cutaneously with H or animals burned mildly or moderately increases in both groups

and apparently represents the effect of skin destruction. However, the adrenals of these scalded animals do not show the marked hypertrophy noted for the H animals.

Severe thermal injury (60 seconds) is responsible for a decrease in the ester cholesterol content which is followed by a tremendous increase during a 2½ day period (Figure 2). The changes after this acute injury occur earlier than those noted after the injection of mustards.

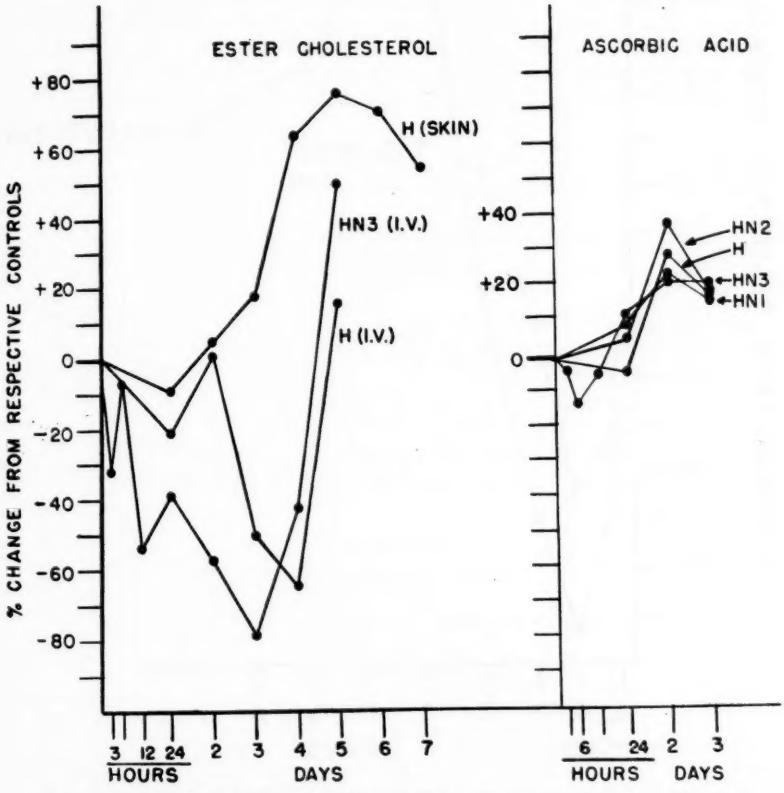

FIG. 1. Effect of Vesicants on the Adrenal Ester Cholesterol and Ascorbic Acid.

The percentage changes from the controls for adrenal ascorbic acid of mustard-injected and for scalded animals are shown in Figures 1 and 2. Following the intravenous injection of lethal doses of mustards, the ascorbic acid content increases and remains high. No similarity is seen between the changes for ascorbic acid and ester cholesterol. After severe thermal injury the marked decrease in ascorbic acid content seen during the first 12 hours is followed by a rapid increase. The pattern closely parallels that observed for the ester cholesterol con-

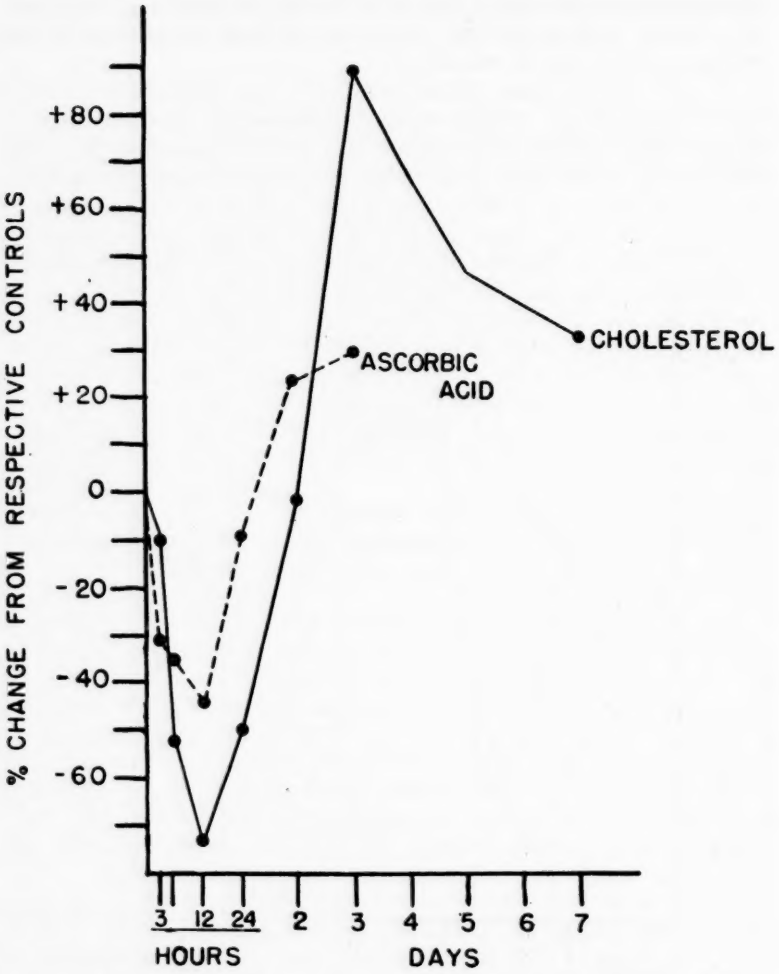

FIG. 2. Effect of Severe Thermal Injury on the Adrenal Ester Cholesterol and Ascorbic Acid.

tent. A similar change is noted for ascorbic acid during the first 24 hours following the injection of adrenotrophic hormone and after hemorrhage (Long, 1946).

SUMMARY

Sodium pentobarbital anesthesia in control rats causes a significant hypertrophy of the adrenals within a few hours. The adrenal ester cholesterol content is significantly decreased by the anesthetic

during the first 6 hours; the free cholesterol content is not affected. The adrenal ascorbic acid content is depressed within a few hours after anesthesia and increases above normal within 24 hours.

Severe thermal injury causes a marked decrease in adrenal ester cholesterol content during the first 24 hours which is followed by a persistent and marked increase after this time. The ascorbic acid content follows a similar pattern. Moderate or mild burns cause significant increases in the cholesterol and ascorbic acid contents. The free cholesterol content is not affected.

After the intravenous injection of tris (β chloroethyl) amine, the adrenal ester cholesterol decreases significantly during the first 12 hours. The ascorbic acid content does not decrease significantly during this period.

Intravenous injections of 3 nitrogen mustards and sulfur mustard cause significant increases in the adrenal ascorbic acid contents after 24 hours.

Cutaneous application of sulfur mustard is responsible for marked adrenal hypertrophy. The ester cholesterol is increased after the third day.

The delayed increases in the adrenal cholesterol and ascorbic acid contents appear to be manifestations of the adaptation syndrome following injury.

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THE EFFECT OF DIETHYLSTILBESTROL ON THE ANTERIOR HYPOPHYSIS OF THYROIDECTOMIZED RATS

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In summarizing the changes in the anterior hypophysis induced by hyperthyroidism, Severinghaus (1942, p. 374) has made the following statement: "Strangely enough the basophiles are again increased in size and number and vacuolation occurs, but to a more limited extent [than after thyroidectomy]. The acidophiles on the other hand show increases in size and number, a greater affinity of their granules for plasma stains, and a definite hypertrophy of the Golgi apparatus which we have agreed to regard as an indication of increased secretory activity." The portion of this characterization which pertains to the acidophiles also summarizes the changes which we (Baker and Everett, 1944) observed in the acidophiles following treatment of immature rats with small doses of diethylstilbestrol administered for brief periods of time. Hence, we were led to suspect that this estrogen might have modified the hypophysis indirectly through a stimulation of thyroid activity. The well-known severe reduction of acidophiles which follows thyroidectomy in the rat gives added strength to our suspicion. The primary purpose of the study being reported was to examine this possibility and constitutes one phase of our present investigative program which aims to define more precisely the action of gonadal hormones on the anterior hypophysis.

The experiment was carried out first on immature rats but, for reasons to be pointed out, the results were not conclusive and it was decided to repeat the entire experiment on adult rats. An additional stimulus for transferring the study to adult rats was derived from the work of Pomerat (1941) and Hunt (1942) who have shown that the mitotic activity of the anterior hypophysis declines with advancing age, and of Hunt (1947) who demonstrated that the capacity of estrogen to stimulate this mitotic activity also decreases as rats become older. Since mitotic division of acidophiles was found to be a major factor in their increase in immature rats treated with diethylstilbestrol, we desired also to examine the action of this estrogen on the hypophysis of adult rats in order to discover whether or not aging of the rat might impair the capacity of estrogen to increase the number of acidophiles.

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Regardless of the hypothesis that estrogen might stimulate the acidophiles of the rat through the thyroid gland, there still remains the possibility that the influence of the thyroid hormone on the oxidative processes of cells in general might modify the action of estrogen on hypophyseal cells through an alteration of the capacity of these cells to respond. For example, in the case of the action of gonadotropic hormones on the gonads, experiments of various types have suggested an inhibitory effect of the thyroid hormone on gonadal stimulation by these hypophyseal gonadotropins (Schockaert, 1931; Fluhman, 1934; Tyndale and Levin, 1937; Smelser, 1939), although Leonard and Hansen (1936) questioned this conclusion. In the light of such evidence one might reasonably expect that thyroidectomy would augment the stimulative action of estrogen on the hypophysis.

MATERIALS AND METHODS

The study was divided into two parts, the first dealing with immature rats and the second, with adult rats (Long-Evans strain). The members of each litter of immature animals were divided into the following groups: (1) oil-treated; (2) stilbestrol-treated; (3) thyroidectomized, oil-treated; and (4) thyroidectomized, stilbestrol-treated. Thyroidectomies were performed at 12 days of age, injections of oil or stilbestrol (2 μg. daily in 0.05–0.1 cc. of sesame oil) were begun on the twenty-fifth day and the rats were sacrificed on the thirtieth day of life. During the first portion of this study, the cervical region of the thyroidectomized animals was examined at autopsy with a binocular microscope for thyroid remnants and suspicious pieces of tissue were studied histologically. Later, the entire midcervical region was sectioned and examined microscopically. At this time it was discovered that numerous animals still retained thyroid tissue. Since all of the thyroidectomized rats showed the usual symptoms of thyroid deficiency, i.e., retarded growth rate, scraggly and dry hair, sluggish activity and a blunted nose, both the completely and incompletely thyroidectomized rats are grouped together and considered as hypothyroid animals.

The members of each litter of adult rats (ranging in age from 153 to 376 days) were divided into the following groups: (1) ovariectomized, oil-treated; (2) ovariectomized, stilbestrol-treated; (3) ovariectomized, thyroidectomized, oil-treated; (4) ovariectomized, thyroidectomized, stilbestrol-treated. Ovariectomy and thyroidectomy were performed at the same operation and hormone treatment was begun after a minimum post-operative period of six weeks. Stilbestrol[1] was given in doses of 2 μg. daily for ten days to the treated rats and comparable amounts of the oil solvent (0.05–0.1 cc.) to the controls. Serial sections of the entire mid-cervical region from the upper border of the thyroid cartilage to the superior mediastinum were examined for thyroid remnants.

At autopsy, the hypophyses were weighed in the fixative and the uteri were weighed after expression of their contained fluid. The marked augmentation in uterine weight in the estrogen-treated animals (table 1) gives proof of the adequacy of the stimulation, while the low uterine weights in

[1] We wish to thank Dr. Erwin Schwenk of the Schering Corp., Bloomfield, New Jersey, for supplying us with diethylstilbestrol.

the non-treated controls shows that they were free of contact with the hormone. The staining techniques and counting procedures applied to the hypophyses were the same as those previously described (Baker and Everett, 1944). The glands were stained with a modified Masson technique[2] after fixation in Bouin's fluid to remove the mitochondria. A differential count was made on every tenth oil immersion field of each of three sections taken from three equally-spaced zones through the gland (in the adults, alternate rows were considered). The quotient of the number of mitoses observed, divided by the total number of oil immersion fields in the above three sections, was taken as an empirical index of mitotic activity. These observations were made with a 90× oil immersion objective and 12× compensating oculars. The average number of cells counted per gland in the immature rats was 1,981 and in the adults, 1,752. The average number of fields observed for mitoses was 158 in the young rats and 139 in the adults.

RESULTS

(a) Immature rats

Bodily weight. Table 1 shows that the bodily weight of non-operated animals continued to increase at the normal rate in spite of the stilbestrol injections thus demonstrating that we were using a dosage of hormone which did not exert significant toxic effects.

As was to be expected, a state of thyroid deficiency retarded the rate of gain in bodily weight over the eighteen day period of the experiment. These rats also gained in weight on the stilbestrol treatment although the magnitude of the increase was less than half that of the non-operated animals. Since these rats were growing more slowly because of a reduced secretion by the thyroid gland, it seems possible that their diminished weight gain can be explained more logically on this basis, rather than on the basis of an increased sensitivity to estrogen.

Hypophyseal weight. In confirmation of our earlier work, it was shown that 2μg. of stilbestrol given daily for four days would cause a considerable increase in hypophyseal weight. In this experiment the hypophysis increased from a mean of 2.0 mg. in the controls to 2.59 mg. after injection of the hormone (table 1). Although there was some variation in the weight of the hypophysis between litters, within a given litter, the hypophysis of the stilbestrol-treated animals was heavier than that of the control in every instance.

A state of thyroid deficiency likewise resulted in an increased hy-

[2] In view of the current disagreement among investigators regarding identification of the various cellular types, it is necessary to point out that during the counting procedures, chromophiles were classed as such solely on the basis of the granulation. In the case of the acidophiles, the granules were stained red by the acid fuchsin and ponceau de xylidene and those of the basophiles, blue by aniline blue. The authors are fully aware that aniline blue does not necessarily reveal true basophilia. However, the employment of triacid dye combinations to delineate the hypophyseal cells has become so deeply ingrained in the American literature, that in our opinion it would be premature to attempt a re-definition of these cells at the present time.

pophyseal weight, the mean being 2.45 mg. as contrasted to 2.0 mg. for the controls. However, this increase did not occur consistently in all individual cases. Stilbestrol treatment of hypothyroid rats led to still heavier hypophyses, the average being 3.26 mg. (table 1).

Mitotic activity. Stilbestrol treatment of non-operated animals increased their mitotic index from .047 for the controls to .189 (table 1) which agrees with our previous findings except that the index here is somewhat lower in the non-treated animals. In contrast to the situation in adult rats, there is considerable cellular division of acidophiles

TABLE I. THE EFFECT OF DIETHYLSTILBESTROL ON BODILY, HYPOPHYSEAL AND UTERINE WEIGHT, AND ON MITOTIC ACTIVITY

Treatment		Mean Body Weight (gm.) Start	Finish	Mean Weight Hyp. (mg.)	Mean Weight Uterus (mg.)	Mean Mitotic Index Acid.	Baso.	Chromo.
Immature rats								
Nonoperated	C	44	52	2.0±0.26[1]	24	.047±.029	.008±.010	.059±.031
	S	42	51	2.59±0.43	105	.189±.118	.004±.005	.069±.026
Hypothyroid	C	37	40	2.45±0.48	31	.006±.010	.006±.005	.034±.028
	S	37	41	3.26±0.72	94	.086±.072	.016±.008	.058±.032
Adult rats								
Ovariectomy	C	256	274	10.38±1.66	82	.003±.005	.003±.002	.001±.002
	S	258	239	13.9 ±3.03	313	.039±.022	.005±.010	.029±.066
Ovariectomy Inc. thyroidectomy	C	241	233	12.7 ±3.0	99	0	.001±.002	0
	S	239	227	18.1 ±1.6	316	.014±.012	0	.002±.003
Ovariectomy Com. thyroidectomy	C	252	253	12.0 ±2.7	109	0	.004±.003	.002±.002
	S	235	221	17.1 ±1.94	312	.068±.055	.001±.002	.002±.004

[1] =standard deviation.
C =control rats.
Com. =complete.
S =stilbestrol-treated rats.
Inc. =incomplete.

in the hypophyses of normal growing rats. In view of the marked reduction in number of acidophiles which follows thyroidectomy, it is of considerable interest that following this operation, the mitotic activity of these cells was stopped almost completely, the mean index being .006. Treatment of thyroid-deficient rats with the estrogen increased the mitotic index to .086. Although this index is not as great as that of the non-operated treated rats, still it represents a much greater percentage increase over the condition of the controls, stilbestrol causing an approximately fourteen-fold increase in the mitotic activity of hypothyroid rats as contrasted to a four-fold increase in the non-operated rats.

Neither thyroidectomy nor stilbestrol treatment of non-operated or thyroidectomized rats altered significantly the mitotic activity of the basophiles, these cells normally exhibiting very little evidence of proliferation.

In both the non-operated and thyroidectomized rats, stilbestrol injections caused some increase in the number of mitoses in the

chromophobes (table 1). Insofar as the intact rats are concerned this result is in agreement with our previous report.

Cellular percentages. Stilbestrol treatment caused an increase in the mean percentage of acidophiles in intact rats, the count being 37.0 as compared with 24.5 for the controls (table 2). A similar increase from 7.7 to 19.7 per cent occurred in the hypothyroid animals.

Although the reduction in number of basophiles induced by stilbestrol in both the intact and thyroidectomized rats was not great,

TABLE II. THE EFFECT OF DIETHYLSTILBESTROL ON THE DISTRIBUTION OF CELLULAR TYPES

Treatment		No. rats	Mean percentage of cells			
			Acidophiles	Basophiles	Chromophobes	Vacuolated
Immature Rats						
Non-operated	C	11	24.5±8.5[1]	11.8±2.5	63.4± 7.2	
	S	10	37.0±9.2	8.0±2.3	54.6± 9.28	
Hypothyroid	C	13	7.7±7.2	11.9±4.2	79.4± 9.5	
	S	18	19.7±9.8	11.7±3.9	67.6± 9.2	
Adult Rats						
Ovariectomy	C	6	32.9±6.5	5.0±1.4	61.9± 7.7	0.02±0.16
	S	7	70.8±2.7	3.4±0.6	25.4±2.8	0.18±0.17
Ovariectomy, Inc. thyroidectomy	C	8	3.7±4.2	6.3±1.7	90.1± 3.6	0.69±0.55
	S	10	38.5±8.7	5.5±2.5	50.7±10.8	0.63±0.36
Ovariectomy, Com. thyroidectomy	C	6	1.2±1.2	5.7±1.4	92.9± 0.7	0.63±0.44
	S	8	43.6±6.2	5.5±2.9	50.7± 3.8	0.37±0.47

[1] Standard deviation.

there was nevertheless a significant degranulation and reduction in size of many of these cells.

The changes in percentage of chromophobes reflected chiefly the alteration in number of acidophiles.

(b) Adult rats

Bodily weight. In contrast to the continued gain in weight of non-treated rats after ovariectomy from a mean weight of 256 gm. to 274 gm., the rats given a total of 20 μg. of stilbestrol over a period of ten days, rather consistently showed a weight loss, the average being from 258 to 239 gm. (table 1). A weight loss of somewhat similar magnitude followed stilbestrol injections into thyroidectomized animals but it should be noted that the incompletely thyroidectomized controls lost weight without hormonal treatment and that the weight of the completely thyroidectomized controls remained static.

Hypophyseal weight. Estrogen treatment increased the weight of the hypophysis from an average of 10.38 mg. for the controls to 13.9 mg. In every instance the hypophysis of the estrogen-treated rat was heavier than that of its control.

Thyroidectomy also resulted in hypophyses heavier than those of the ovariectomized controls. These glands in the incompletely thyroidectomized animals weighing on an average 12.7 mg. and in the completely thyroidectomized rats, 12.0 mg. However, the increase in weight in individual cases was much less regular and consistent than after stilbestrol treatment of intact animals. Estrogenic stimulation further increased pituitary weight, in the incompletely thyroidectomized group, the mean being 18.1 mg. and in the completely thyroidectomized rats, 17.1 mg. (table 1).

The average number of cells counted per field in the ovariectomized controls was 118 as contrasted to 95 after estrogen treatment which indicates enlargement of some of the cells, these being the acidophiles. In the ovariectomized-incompletely thyroidectomized and -completely thyroidectomized groups, these average counts were 109 and 118, respectively, which indicates no very great change in general cellular size due to thyroidectomy over the ovariectomized condition. Here also, after estrogen treatment the number of cells counted per field was reduced to 90 and 95 for the incompletely and completely thyroidectomized groups, respectively, showing that in the hypothyroid state, diethylstilbestrrol is still able to induce cellular hypertrophy.

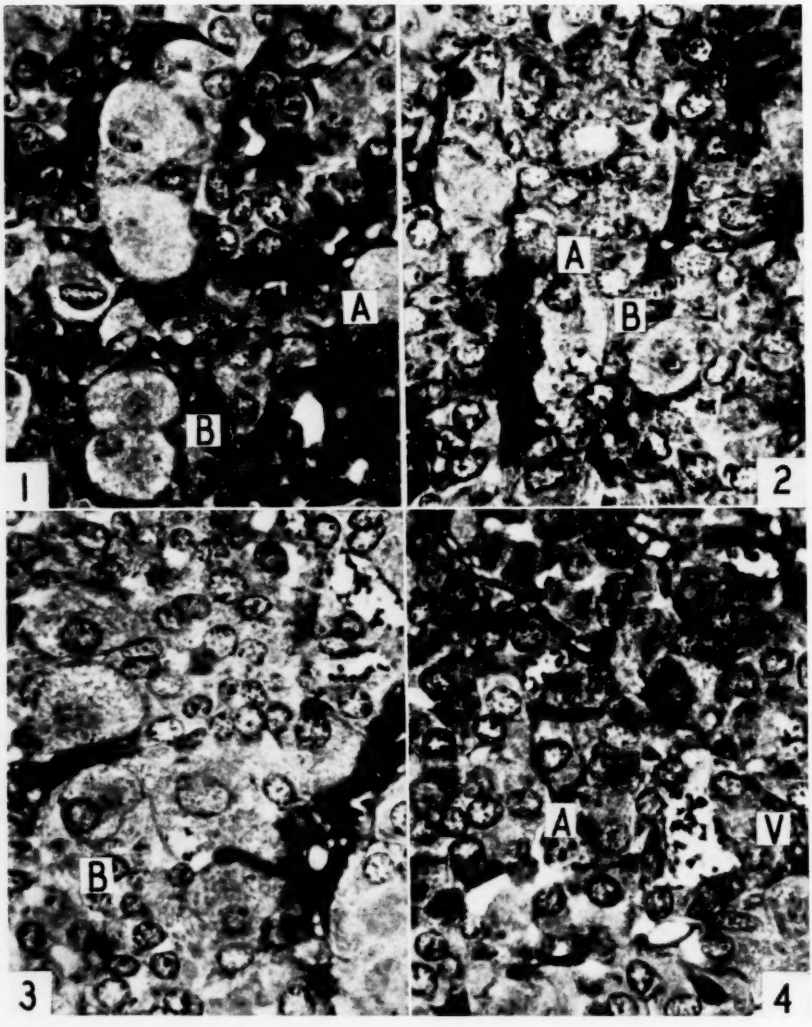
Mitotic activity. In contrast to the situation in immature rats, the mitotic activity of all cells in the hypophyses of adult ovariectomized rats is negligible. In the acidophiles of our specimens, the average index was .003. Stilbestrol increased this index to .039 (table 1). The acidophiles of both the incompletely and completely thyroidectomized controls exhibited a mean index of 0 which was increased by estrogen in both groups, the mean index being .014 for the former and .068 in the latter.

Neither thyroidectomy nor stilbestrol noticeably modified the already negligible mitotic activity of the basophiles.

Stilbestrol treatment stimulated the chromophobes of ovariectomized rats to accelerated division, the index being increased from .001 to .029. Significant stimulation by this means did not occur after thyroidectomy (table 1).

Cellular percentages. The stimulating effect of estrogen on the acidophiles of the adult under the conditions employed was even more striking than in the immature rat. The mean percentage of acidophiles in the ovariectomized controls was 32.9. This figure was increased by stilbestrol to 70.8 (table 2). The rather small standard deviation of 2.7 for the injected rats indicates how uniform this response really was.

Complete thyroidectomy for a period of over seven weeks almost



EXPLANATION OF FIGURES

The hypophyses illustrated are from individuals of the same litter. Bouin fixation, 3μ, modified Masson stain.

FIG. 1. Ovariectomized, oil-treated. Two large basophiles are at the left of B and a smaller one at the right. Most of the basophiles are large and are densely granulated. Most of the acidophiles (cluster of black cells below A) are diffusely and densely granulated. Percentage of acidophiles: 31.3%.

FIG. 2. Ovariectomized, stilbestrol-treated. Basophiles (to the right and left of B) are smaller and less densely granulated than in figure 1. The majority of the cells in this area contain somewhat diffusely scattered acidophilic granules (black dots) as in the cell above A where the granules may be seen at the right of the nucleus. Percentage of acidophiles: 73.6%.

completely removed the acidophiles, the mean percentage for these cells being 1.2. The retention of small fragments of thyroid tissue did not alter this response very much, the mean percentage for this group being 3.7. Treatment of both the incompletely and completely thyroidectomized groups with estrogen induced a marked increase in the number of acidophiles, the average percentages for these two groups being 38.5 and 43.6, respectively, which represents a higher level for this cellular type than that of the ovariectomized non-injected group. Here again, the presence of small pieces of thyroid tissue did not affect very noticeably the response of the acidophiles to estrogen.

Seven weeks after ovariectomy, the acidophiles were of varied form, many tending to be spherical but a large number were stellate (fig. 1). Most of them were diffusely granulated and stained with varied intensity. In most of these cells individual granules were not discretely visible. Only a small proportion of them was sparsely granulated with large Golgi regions. After stilbestrol treatment the picture changed considerably. The vast majority of the acidophiles were then of moderate size being considerably smaller than the large basophiles. The granules stained a brilliant red, were coarse and sharply defined (fig. 2). They were sparsely distributed but tended to aggregate in dense clumps at the periphery of the cell especially at the side opposite to the eccentrically-placed nucleus (fig. 4). The orientation of the granules bore no constant or striking relationship to the sinusoids. The Golgi region was large and seemed to extend through a considerable portion of the cytoplasmic space. It stained moderately dark with aniline blue but less intensely than did the granules of the basophiles. A few red granules were generally present in this region. The vesicular nuclei of most of these cells contained rather large nucleoli.

Except for numbers, the cytology of the acidophiles which appeared after estrogen treatment of ovariectomized-thyroidectomized animals was similar to that of the acidophiles of the ovariectomized-treated rats (figs. 3, 4). However, there reappeared none of the fully granulated acidophiles which had been practically wiped out during the post-operative period following thyroidectomy.

No non-operated controls were available to enable one to determine the extent to which ovariectomy itself increased the number of

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FIG. 3. Ovariectomized, thyroidectomized, oil-treated. The basophiles are generally large and densely granulated as in the three cells above B. Some cells are vacuolated as shown in the upper right hand corner. No acidophiles are visible in the field. Percentage of acidophiles: 0.35%.

FIG. 4. Ovariectomized, thyroidectomized, stilbestrol-treated. The field does not show basophiles well. Vacuolated cells are still present as shown at the left of V. Many acidophiles are present. The one above A illustrates the typical eccentric position of the nucleus, hypertrophy of the Golgi area located below the nucleus and aggregation of the dark granules at the periphery of the Golgi region. Percentage of acidophiles: 38.5%.

basophiles. The mean percentage of 5.0 per cent represents only a very slight increase over the number which we observed during lactation (Everett and Baker, 1945). However, it should be noted that the majority of the basophiles present were very large and densely granulated (fig. 1). Incomplete and complete ablation of the thyroid combined with ovariectomy resulted in only slight additional increases, the mean percentages being 6.3 and 5.7, respectively. Here, again, the basophiles were very large and densely granulated (fig. 3). Stilbestrol treatment in all three operated groups (table 2) caused some reduction in the number of basophiles and a more significant reduction in size and granulation of these cells.

As in the immature rats, the number of chromophobes in the various groups reflected chiefly the changes in the number of acidophiles.

Only a small number of vacuolated cells were observed seven weeks after ovariectomy, the mean percentage being 0.02 while after stilbestrol treatment the average was 0.18. This increase cannot logically be considered significant but it does demonstrate that at the dosage used, stilbestrol did not correct the vacuolated condition of these cells. The combination of thyroidectomy with ovariectomy produced a larger number of vacuolated cells, the mean percentage being 0.69 and 0.63 for the incompletely and completely thyroidectomized groups, respectively. Stilbestrol treatment in the incompletely thyroidectomized group left the count practically unchanged while in the completely thyroidectomized rats it was reduced to 0.37. The latter reduction suggests a tendency toward repair but it can be concluded that at this dosage level, which so significantly stimulated the acidophiles, there had occurred very little correction of the vacuolated state of some of the pituitary cells.

DISCUSSION

The study of the action of estrogen on the hypophysis of thyroidectomized rats was undertaken initially on immature rats in order to make possible a correlation of the results obtained with those of our earlier work which had been carried out on young animals. A significant stimulating effect of stilbestrol on the acidophiles was observed, but there was considerable variation between litters in the number of acidophiles found both before and after treatment with stilbestrol. It seemed that two major factors may have accounted for this situation. First, the thirteen-day span from the time of thyroidectomy to the institution of injections may have been an inadequate period in which to allow for complete disappearance in all cases of the animal's circulating thyroid hormone. It was not possible to extend this period because some of our rats establish vaginal introitus as early as 33+ days of age and soon thereafter run estrous cycles. In such cases, the cyclic alterations in the endogenous estrogen level would have introduced an uncontrollable variable into the experiment. Second, micro-

scopic search for thyroid remnants had not been pursued in all animals, and since pieces were found in a high percentage of the rats in which it was done, we suspected that retained bits of thyroid tissue may have been responsible for the variation in results. Consequently, the study was repeated in adult rats from which the ovaries had been removed, with a minimum post-operative period after thyroidectomy of six weeks and in which a microscopic search for thyroid remnants was made.

It is evident from our results that with proper adjustment of dosage and duration of treatment, it is possible to induce a marked increase in the number of acidophiles in adult rats. Just as accelerated mitotic activity in young rats plays a pre-eminent rôle in the increase of these cells under estrogen treatment, so also in adult rats, hyperplasia of the acidophiles is stimulated sufficiently and consistently so that it must be considered here, also, to play an important part in their multiplication. Nevertheless, one cannot exclude the possibility that conversion of chromophobes to acidophiles under the influence of estrogen also occurs. In summarizing the manner in which cells of the hypophysis alter their relative numbers Severinghaus (1938) has stated that "The scarcity of mitotic divisions rules out the possibility that specific cells multiply sufficiently to change the relative cell proportions. They must then change from one specific cell type into another." Our work suggests that this position requires qualification in regard to the estrogenically-induced increase in the number of acidophiles in immature rats and that it must be modified in connection with their increase in adult rats also.

All previous studies of the effects of various estrogens on the acidophiles of the adult female rat hypophysis have resulted in either no change, an "erratic reaction," a "greater resistance to degranulation than the basophiles," or a reduction in number (Zondek and Berblinger, 1931; Baniecki, 1932; Bachner, 1933; Nelson, 1934; Hohlweg, 1934; Desclin, 1935; Wolfe, 1935a, 1936; Halpern and D'Amour, 1936; Foster, 1942). With the exception of Wolfe, none of these investigators reported differential cell counts. Wolfe (1935b) observed a decrease in the relative number of acidophiles following estrin injections but thought that there was probably an increase in their total absolute number. Of pertinence is his observation that many of the acidophiles were undergoing cell division. It would appear that the chief reason why an increase in the acidophiles was not observed in most of these studies was due to the fact that too much estrogen was injected for too long a period of time, thus causing a degranulation of the acidophiles. In our previous study (Baker and Everett, 1944) it was found that the daily injection of 0.5 μg. of stilbestrol into immature rats caused an increase in the acidophiles if the period of treatment lasted for 7 days but if this period were extended to 14 days a reduction was noted.

The significance of this effect of a synthetic estrogen on the acidophiles is enhanced by the demonstration that naturally occurring estrogens will induce similar increases. This has been demonstrated in the immature rat by Meyer, Biddulph and Finerty (1946) with estradiol and in the adult ovariectomized rat with alpha-estradiol dipropionate (Piper and Baker, unpublished).

A number of investigators have studied the effects of various estrogens on the hypophyses of thyroidectomized rats and insofar as the acidophiles are concerned, they all reported a failure of estrogen to restore the acidophiles to the hypophysis. An attempt should be made to explain the discrepancy between their results and ours. It appears here also that the major reason why others may not have detected the increase in acidophiles was because they injected a sufficiently large dose of estrogen for a long enough period of time to cause an extensive degranulation of these cells thus converting them to chromophobes. Most of these investigators were interested primarily in injecting sufficient estrogen to prevent or repair the vacuolated cells which our study has shown must occur at a higher dosage level than that necessary to stimulate the acidophiles. Nelson and Hickman (1937) used adult male and female rats which had been gonadectomized or gonadectomized and thyroidectomized for 42 days or more. The minimal treatment which they administered was 40 R. U. of estrone daily during the last 20 days of the 42 day period. They concluded: "A marked decrease in the number of granulated acidophiles was present in all animals and was apparently unaffected by oestrone-treatment." A subsequent report by Nelson (1942) also confirms the lack of effect of estrogen on the acidophiles. Leblond, Albert and Selye (1942) treated adult male rats daily with .05 mg. of alpha-estradiol and ethinyl estradiol (other steriods were used also) for 22 days after ovariectomy. This dosage prevented the appearance of vacuolated "thyroidectomy" cells but further degranulated the acidophiles so that they concluded the degranulating effect of the operation and estrogen treatment to be additive.

Little information can be derived from our studies regarding the effect of age on mitotic activity. They do indicate that when the endogenous source of estrogen is removed by ovariectomy that there are practically no mitoses present in the anterior hypophysis. As for the comparative responsiveness of the hypophysis of young and mature rats to estrogen, it is also impossible to draw a precise conclusion from our results because the total dosage and duration of the experiment were not the same in both cases. Nevertheless, we have demonstrated that in the adult rat one can develop with estrogen treatment a greater relative number of acidophiles than in the immature rat where prolongation of the injection period resulted in degranulation and reduction in number (Baker and Everett, 1944). It is clear also that in the adult rat, stilbestrol accelerates hyperplasia of the acidophiles.

Others (Nelson and Hickman, 1937; Leblond et al., 1942) have reported that estrogen injections will prevent or repair the vacuolation of "thyroidectomy" cells, although Zeckwer (1938) who differentiated between the vacuolated cell of ovariectomy and thyroidectomy, stated that estrone in total doses ranging from 595 to 4350 I. U. would not repair those which appeared following thyroidectomy. Our problem was not concerned primarily with these cells but our findings do demonstrate that acidophilic stimulation occurs at a dosage level below that necessary to remove the vacuolated cells.

Our results are at variance with a large number of workers who have described a marked increase in the number of basophiles after both ovariectomy and thyroidectomy. We agree that there is a striking "basophilia" in both conditions if this conclusion is based on the generally large size and dense granulation of the basophiles. However, our counts failed to reveal a very great increase in the number of basophiles. It might perhaps be noted that many of the previous reports have not been based on differential cell counts and that without actual counts one may easily gain a mistaken impression of the relative numbers of cellular types in the anterior hypophysis. A more likely explanation for this discrepancy may be found in the type of fixative and stain which we have used. Most reports dealing with basophilia in these conditions have been based on material fixed in modifications of Zenker's fluid or in Champy's fluid, the former, at least, being one of the best preservatives for ribonucleic acid. True cytoplasmic basophilia in the anterior hypophysis is dependent on the presence of this substance. Since in Bouin fixation and staining with aniline blue we have not used a desirable technique for the demonstration of ribonucleic acid, it is possible that our procedure has not revealed all forms (or types?) of basophiles. If this explanation is correct, it will not detract from the accuracy of our findings in regard to the relative numbers of acidophiles because such poorly fixed "basophiles" would appear in our counts as chromophobes. Whatever the ultimate solution of this problem may be, it must be emphasized that the technical procedure we have employed differentiates basophiles (with the structure illustrated in fig. 1) from the other basic cellular types with a precision and sharpness of definition which can be equalled with few other methods.

There is almost complete agreement among investigators that hypothyroidism (for reviews of the literature see Simpson and Hunter, 1911; and Marine, 1922) and estrogen treatment increase the weight of the hypophysis. Our experiments show that the two states existing together may exert an augmentative effect on pituitary weight. Beyond a general hypertrophy of the basophiles, our observations did not offer any additional explanation for the hypophyseal hypertrophy of hypothyroidism. However, seven weeks after thyroidectomy, we found this glandular enlargement to be erratic. The

estrogen-induced hypertrophy is more satisfactorily explained by the acidophilic hypertrophy and hyperplasia.

Finally, it may be concluded from our study that with low doses of diethylstilbestrol one may cause a marked increase in the relative number of acidophiles in the adult rat hypophysis, that this action of estrogen is not mediated by the thyroid gland and that accelerated mitotic activity of the acidophiles plays an important part in bringing about this increase.

Further, for a number of reasons, we have gained the impression that the acidophiles may represent the primary point of estrogenic stimulation in the anterior hypophysis. First, in all hypophyses of estrogen-treated rats which we have studied, stimulation of mitotic activity was always greater in the acidophiles than in the chromophobes. Second, in all but one adult ovariectomized rat, this accelerated mitotic activity occurred in the acidophiles to the complete exclusion of the chromophobes and, similarly, after thyroidectomy, the acidophiles and not the chromophobes were stimulated to divide. Third, using the technique for preparation and criteria for identification as previously outlined, we have never observed any indication of stimulation of basophiles other than degranulation which might also be interpreted as having resulted from inhibition of these cells.

SUMMARY

The injection of 2 μg. daily of diethylstilbestrol into immature thyroidectomized rats for four days or into adult ovariectomized-thyroidectomized rats for 10 days caused an increase in weight of the hypophysis, cellular enlargement, an accelerated mitotic activity among the acidophiles and an increase in their number. Thus, it was demonstrated that this stimulating action of estrogen on the hypophysis is not mediated by the thyroid gland and that it may be elicited in adult as well as in immature rats.

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A BIO-ASSAY FOR MINUTE AMOUNTS OF PROGESTERONE[1]

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THE ENDOMETRIUM of the mouse exhibits an apparently specific response to progesterone that consists primarily in hypertrophy of the stromal nuclei (Hooker, 1940, 1945). Since the response to progesterone in this species is much simpler morphologically than the complex glandular changes in the rabbit, since the systemic dose required is less (0.125 mg. as compared with 0.5 mg.), and since the amount of tissue exposed to the hormone upon direct administration is necessarily small, local administration in the mouse offered promise of providing a highly sensitive method of assay. As will be described, the method proved feasible and regularly detects progesterone in an amount of 0.0002 μg.

EXPERIMENTS

Technique. To minimize variations in the test animals, all of the 200 mice used have been members of the highly inbred CHI strain of Strong (1942), maintained on a diet of Purina Fox Chow and water supplied ad libitum. This strain exhibits no peculiarities in response or sensitivity to progesterone (Hooker, 1945). Inasmuch as the response consists primarily of nuclear hypertrophy, the tissues for microscopic study were fixed in Lavdowsky's fluid (Williams and Hodge, 1943), which produced less shrinkage than other fixatives tested. Paraffin sections 6 micra in thickness were stained with Harris' hematoxylin and eosin.

It immediately became apparent that intra-uterine injection of volumse as large as 0.01 cc. produced distention of the uterine segment frequently sufficient to obliterate the tunica propria. It was necessary, therefore, to employ an apparatus that would make possible the accurate injection of much smaller volumes. The instrument used (Fig. 1) consists of a micrometer caliper head mounted on a brass base to which a one-half cc. tuberculin syringe is clamped by phosphor-bronze clips. The syringe is readily removed for cleaning and filling. The plunger of the syringe is moved forward by ad-

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Miss A. Jayne Foley gave excellent technical assistance.

vancement of the micrometer bolt. The device, exclusive of syringe and needle, weighs 63.5 gms.[2]

The apparatus was calibrated by filling the syringe with mercury and delivering and weighing numerous measured quantities. The graduations on the syringe were disregarded. It was found that advancing the micrometer bolt 0.001 inch regularly delivered 0.0003 cc. (actually 0.0002956 cc.). It has not been necessary to use smaller volumes, and the volume-equivalents of the vernier scale have not been determined.

Two additional difficulties had to be overcome before intra-uterine injections were satisfactory. It was highly desirable that the test segment of uterine horn be of uniform length. It was necessary also to prevent leakage

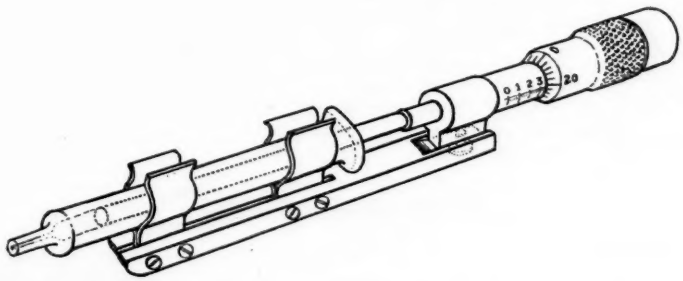


FIG. 1. Apparatus for intra-uterine injections. See text for details.

of injected material through the opening in the uterine wall made by the needle of the syringe. These objectives were attained by use of the following technique.

In a test animal anesthetized with Sodium Amytal supplemented with ether, one uterine horn is delivered through a mid-ventral incision and anchored by a needle passed through the mesometrium (Fig. 2). A tight ligature is placed near the cranial end of the cornu. A second thread is placed approximately 5 mm. caudal to the first, and a single loose overhand knot is tied. In delivering the horn through the abdominal wall the large vessels that course in the mesometrium parallel to the uterus are identified, and care is taken to place the anchoring needle and the ligatures between these vessels and the horn. This precaution preserves the blood supply to the entire uterus. The ends of both ligatures are passed through two slots 5 mm. apart in a device (a comb suffices) clamped horizontally about one and one-half centimeters above the animal. The slots serve to measure a 5 mm. segment of uterus and to draw the threads vertically. The ends of the threads are gripped in artery clamps attached to a horizontal rod. By means of the threads the horn is elevated slightly above the abdominal wall and held securely.

After making sure that the needle is filled with fluid, the horn is grasped with a forceps about 3 mm. caudal to the posterior ligature, and the needle, bevelled surface up, is inserted into the uterine lumen immediately cranial

[2] The injection device was constructed and in part designed by Mr. A. F. Enersen of the Sloane Physics Laboratory machine shop, Yale University.

to the forceps and pushed forward almost to the anterior ligature. The desired volume of fluid is then discharged into the cornu, and the needle is withdrawn. During withdrawal of the needle tension is placed on the caudal thread sufficient to produce a sharp angle in the horn when the needle is removed. The tension is continuously maintained until the knot is drawn tight. After cutting the threads close to their knots, the second horn is sim-

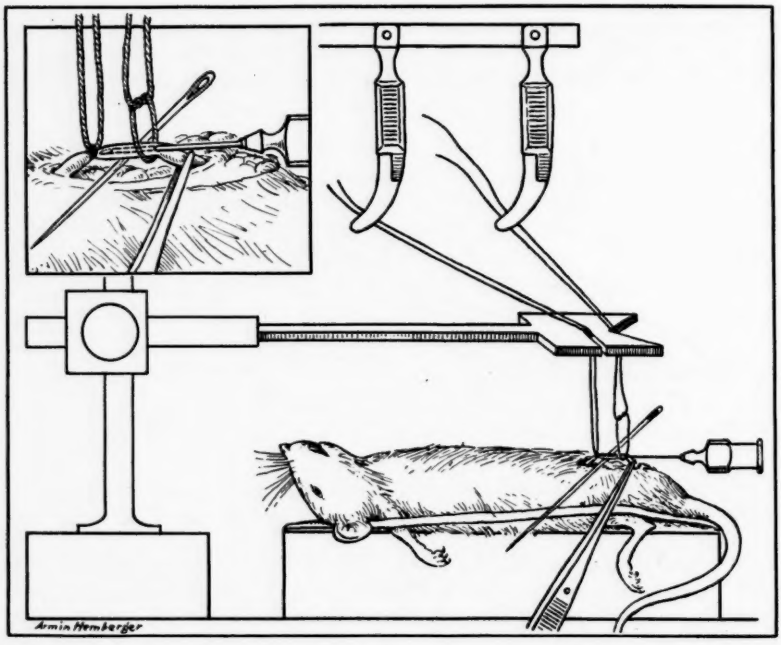


FIG. 2. Method for intra-uterine injection. See text for details.

ilarly injected if desired, the organ is returned to the abdominal cavity, and the body wall is closed with silk sutures. This procedure has repeatedly given injected segments 5 mm. long as measured immediately after injection. At autopsy the segment frequently is shorter as a result of contraction; this shortening, however, should not change the total amount of endometrium exposed to the injected fluid.

Various volumes of sesame oil (the vehicle employed for all of the pure compounds tested) were thus injected into the uteri of ovariectomized mice. It was found that more than 0.0006 cc. produces objectionable distention. Accordingly, 0.0006 cc. was adopted as the standard volume of vehicle. Oil Red O dissolved in sesame oil was observed to fill the lumen of the entire segment, indicating that the full length of the segment is exposed to the fluid injected. The uniform distention revealed by serial sections of the entire segment was a further indication of uniform distribution of the injected oil.

Reasoning from the degree of involution of the stromal nuclei observed

five days after ovariectomy of pseudopregnant mice (Hooker, 1945), it was assumed that a basal condition would be attained in seven days. Repeated tests, however, gave inconstant values for the minimum effective dose of progesterone, and occasionally the condition of the stromal nuclei suggested a positive response to oil alone. Since the lack of uniformity could have resulted from differences in the degree of castration change in the test mice, untreated mice were killed for study at daily intervals after ovariectomy. It was found that 16 days is the minimal interval after ovariectomy which assures that all of the endometrial stromal nuclei of every mouse will exhibit the full castration condition (Fig. 3)—a shrunken, fusiform appearance, with clumped chromatin and no recognizable nucleolus (Hooker, 1945). When the test animals have been given intra-uterine injections of progesterone 16 days after ovariectomy the minimum effective dose has been constant.

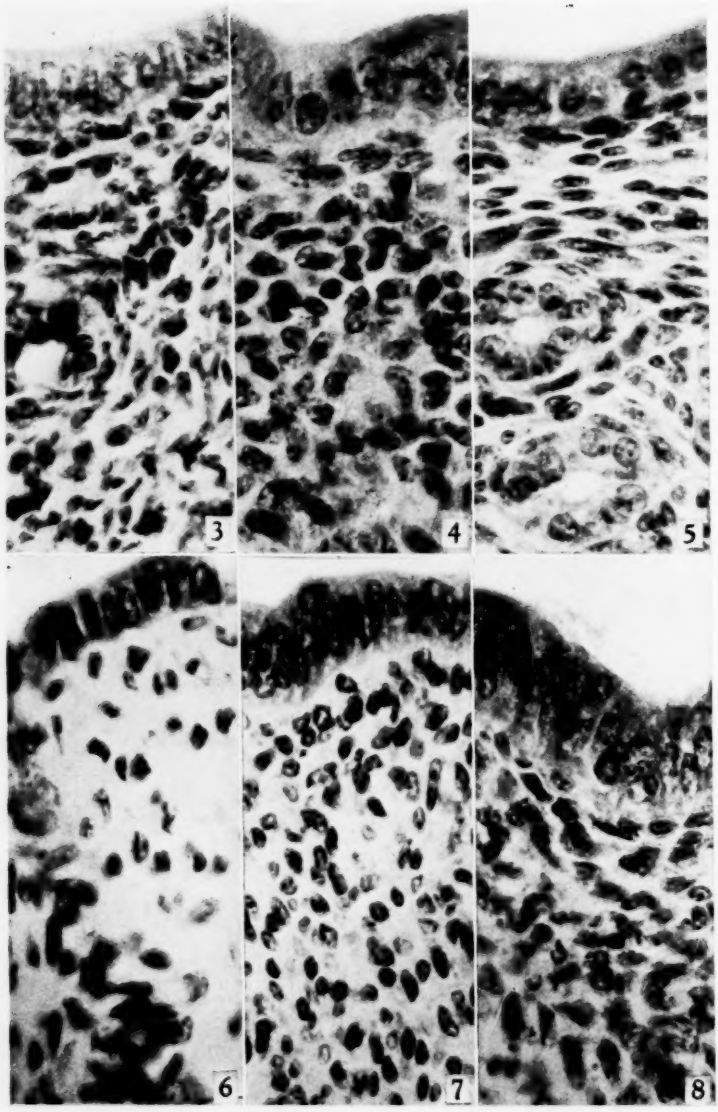
The optimal interval between intra-uterine injection and autopsy was determined in three groups of mice given serial dilutions of a solution of progesterone in sesame oil. The first group was killed six hours after injection; the second group, 24 hours after injection; the third group, 48 hours after injection. The threshold dose was found to be lowest when the interval was 48 hours. When the interval was 24 hours the minimal effective dose was doubled, and when six hours, quadrupled.

The Response. As with subcutaneous administration, the intra-uterine injection of adequate amounts of progesterone results in enlargement of the stromal nuclei of the endometrium (Fig. 4). They assume a smooth, slightly elongated, oval outline. The chromatin particles are fine and evenly distributed, and the nucleolus is conspicuous. It must be emphasized that all of these conditions must be met before the response can be considered positive. It is necessary also to distinguish between stromal nuclei and the nuclei of the glandular epithelium. The latter nuclei may appear to have no cytoplasm, but they can be recognized as belonging to the uterine glands by examining adjacent serial sections. The nuclei of the glandular epithelium do not appear to be modified by progesterone. They are irregularly rounded and have inconspicuous nucleoli and comparatively coarse chromatin particles that are often clumped near the nuclear membrane.

The mesometrial half of the endometrium is distinctly thinner than the antimesometrial portion. When the uterine segment is moderately distended by the injection of 0.0006 cc. of oil the thin portion of the endometrium is frequently stretched sufficiently that stromal nuclei are identified with difficulty. They are, however, readily identified in the thicker endometrium.

For purposes of assay it was decided that the minimal effective dose of hormone would be defined as the least amount which would induce a positive response of any stromal nuclei.

Minimal Effecitve Dose. When the technique of intra-uterine injection is used as described, when CHI strain mice ovariectomized 16



Photomicrographs (×800) of typical sections of endometria of mice ovariectomized 16 days previously.

FIG. 3. No treatment. The stromal nuclei are shrunken and have clumped chromatin.

FIG. 4. After intra-uterine injection of 0.00075 μg. progesterone. A characteristic stromal nucleus is oval and has a conspicuous nucleolus and fine, evenly dispersed chromatin particles.

FIG. 5. After sesame oil alone. The stromal nuclei are spindle-shaped.

days previously are the test animals, and when the interval between injection and autopsy is 48 hours, the minimal effective dose of progesterone dissolved in sesame oil has consistently been 0.0002 μg. That is, no amount less than this has elicited a response in 16 mice, while doses of 0.0002μg. or higher have invariably evoked positive responses. Leakage of injected material from the test segment regularly resulted in false assay values, as would be expected.

Non-injected levels of treated horns as well as the contralateral cornua of unilaterally treated mice were studied microscopically. When less than 0.005 μg. progesterone was given the response was limited to the injected segment. When the amount was 0.005 μg. or higher a response was always seen in the contralateral cornua of unilaterally injected animals.

Specificity. In view of the report (Tobin, 1941) that sesame oil is not always inert, 0.0006 cc. of the oil used as the vehicle was injected under the standard conditions into 6 uterine cornua. Although the nuclei were perhaps slightly enlarged, the characteristic reaction to progesterone was not evoked in any of these uteri (Fig. 5). The possibility that sesame oil and the concomitant mild distention of the test uterine segment might interfere with the responsiveness of the endometrium was examined in one animal. Here 0.0006 cc. sesame oil was injected into a segment of one horn while the other horn was not disturbed. At the time of injection and on the next day the animal was given a subcutaneous injection of 1.0 mg. progesterone. On the following day the animal was killed. No difference in the intensity of the progestational reaction in the two horns was evident.

To determine whether the change in the stromal nuclei after intrauterine injection is a specific response to progesterone, two steroids with progestational (or luteoid) activity were given. Desoxycorticosterone acetate is one of the stronger luteoids (Selye and Masson, 1943) and sometimes, but not always, provokes progestational change upon systemic administration in the mouse (Hooker, 1945). One uterine cornu was injected with each of the following amounts of this compound in sesame oil: 0.6, 3.0, and 6.0 μg. In no instance was a uterine change like that evoked by progesterone seen; the nuclei were irregularly swollen, and the chromatin particles were coarse (Fig. 6). Testosterone is a much feebler luteoid, according to Selye and Masson (1943). Four uterine horns (2 mice) were given this substance, the individual amounts being 0.06, 0.12, 0.24, and 0.48 μg. Here again the endometrium was unlike that seen after effective amounts of progesterone (Fig. 7); this androgen appeared to affect the nuclei in somewhat the same way as did desoxycorticosterone acetate.

FIG. 6. After 6.0 μg. desoxycorticosterone acetate. Edema is conspicuous; the stromal nuclei show little change. Part of a gland appears at the left.

FIG. 7. After 0.48 μg. testosterone. In being smaller and wrinkled, the stromal nuclei fail to meet the criteria for a positive response to progesterone.

FIG. 8. After 0.00075 μg. estradiol. The chief response is heightening of the epithelium. The stromal nuclei are somewhat enlarged.

Inasmuch as estrogen is nearly always associated with progestin, the capacity of estrogens to mimic the effect of progesterone was examined in 33 mice. One animal was given a unilateral intra-uterine injection of sesame oil only and a subcutaneous injection of 6.25 μg. estradiol benzoate. Neither horn showed a modification of the stromal nuclei. Estradiol was given by intra-uterine injection to 29 mice in amounts ranging from 0.00000002 μg. to 0.00075 μg. The stromal nuclei were moderately enlarged but contained coarse chromatin particles, and failed to meet the criteria for the progesterone response (Fig. 8). The largest amount of estradiol given is 3.75 times by weight the effective level of progesterone. Estrone was given by intra-uterine injection to 3 mice in amounts of 0.15, 0.3, and 0.6 μg. Here, also, no progesterone effect on the stromal nuclei was seen.

Estrogen and progesterone were also given simultaneously. In one animal 6.25 μg. estradiol benzoate was given subcutaneously, and 0.0015 and 0.00075 μg. progesterone were given by intra-uterine injection. The reaction to the progesterone was not evidently altered. The uteri of 4 mice were injected with 0.00062 μg., and of one animal with 0.0375 μg., estradiol mixed with amounts of progesterone ranging from 0.000094 to 0.00075 μg. The minimal effective dose of progesterone was 0.0002 μg. just as in animals not given estrogen.

Tests of Unpurified Materials. To determine whether the method is applicable to unpurified materials several tests were made. The first was assay of a crude extract of sows' corpora lutea. From fresh ovaries 336 gm. of corpora lutea were dissected and extracted according to the method of Allen (1932) as far as the ether phase. The material was incorporated in sesame oil and assayed by intra-uterine injection in mice, using serial dilutions of the oily solution. The extract was also assayed in four ovariectomized rabbits according to the method of Allen (1930). The two assays gave identical values for the progestin content of the extract.

A second material was citrated plasma from two rabbits bled on the sixth day of pseudopregnancy. As usual, serial dilutions of the material were injected, the diluent here being 0.9% saline. In both instances the progestin activity in 1 cc. of the plasma was equivalent to that of 4.3 μg. progesterone. For comparison, citrated plasma from a rabbit bled two weeks after ovariectomy was tested. No activity was detected.

A third material was blood serum from mice. Two adult mice of the CHI strain, ovariectomized 33 and 44 days earlier, were given subcutaneously 2.0 mg. progesterone in 0.1 cc. sesame oil. The mice were bled by cardiac puncture 7¼ and 6¼ hours later. Two additional mice, ovariectomized on the same days as the first, were also bled by cardiac puncture. After centrifuging each sample, 0.0006 cc. of each serum was injected into one uterine horn of a test mouse. The serum from the untreated, ovariectomized mice evoked no change in the stromal nuclei, whereas the serum from the progesterone-treated

mice elicited a typical progestational response in the treated cornua but not in the contralateral, uninjected cornua. Accordingly, the progestin activity of 0.0006 cc. of the serum of the progesterone-treated mice was as much as that of 0.0002 μg. of pure progesterone but less than that of 0.005 μg. Hence the activity per cubic centimeter was between 0.33 μg. and 8.25 μg. pure progesterone.

The fourth material was liquor folliculi obtained from the sows' ovaries furnishing the corpora lutea already mentioned. The fluid was aspirated from the follicles, the straw colored fluid from "normal" follicles and the dark amber fluid from cystic follicles being collected separately. Both samples were stored in the refrigerator for several days before being tested. Volumes of 0.0006 cc. and 0.0012 cc. of one sample were injected into the uterine horns of one mouse, and the same volumes of the other sample were injected into the cornua of a second mouse. In both instances the larger volume had so distended the uterine segments that the cellular details could not be evaluated. Both segments receiving the smaller volume exhibited a positive response. Moreover, the endometrium cephalad to all four injected segments showed typical progestational change. Accordingly 0.0012 cc., and possibly 0.0006 cc., of follicular fluid exhibited progestin activity in excess of 0.005 μg. progesterone. This is equivalent to a concentration of 4.13 or 8.25 μg. per cubic centimeter of follicular fluid. The follicular fluid also contained estrogen, as shown by the character of the epithelium of the test uterine segments. This is a further instance of the failure of estrogen to inhibit the reaction of the mouse's uterus to progesterone.

DISCUSSION

The standard method for the assay of progesterone involves subcutaneous administration into the rabbit (Allen, 1930). On the basis of this test one international unit of activity is shown by 1 mg. of the pure compound. Several variations of this method have been advanced (Clauberg, 1930; McPhail, 1934). Although the method and its variants were of incalculable service in the purification of progesterone, their requirement of a relatively large amount of material has limited their application to several biologic problems. Moreover, a positive test is given by a large number of compounds other than progesterone (Selye and Masson, 1943).

When progesterone is injected directly into uterine segments of the rabbit, much smaller amounts have proved effective. Mussio-Fournier et al. (1938) observed a minimal response with 0.1 mg. McGinty, Anderson, and McCullough (1939) reported that 0.5 to 5.0 μg. injected directly into the uterus elicited responses comparable to those elicited by 0.5 mg. when given intramuscularly. Haskins (1939, 1941) observed irregular responses to as little as 0.25 μg. progesterone and to small amounts of serum from pregnant guinea pigs and of ether extracts of blood of pregnant women. Halvorsen (1944) obtained a

minimal response in 4 of 10 animals with 0.0025 μg. and positive responses in 9 of 10 animals with 0.025 μg.

The present test with a standard minimal effective dose of 0.0002 μg. thus appears to be more sensitive than other available methods. Moreover, it apparently possesses the desirable attribute of specificity, as shown by the failure of relatively large amounts of desoxycorticosterone acetate and testosterone, both of which have luteoid properties (Seyle and Masson, 1943) to provoke the response. (The ability of other compounds to mimic the action of progesterone upon intra-uterine injection seems not to have been examined in the rabbit.) In addition to its sensitivity, the test offers the advantage of being less expensive than any of the methods involving rabbits in terms of the cost of the test animals, the space required for their housing, and their food. The use of inbred strains, further, presumably minimizes the variation from animal to animal.

It is interesting to compare the sensitivity of the intra-uterine test for progesterone in the mouse with the most sensitive methods for the assay of other steroid hormones. With respect to the androgens, the international unit is based upon intramuscular injection in capons and is 100 μg. for androsterone and 13–16 μg. for testosterone (Koch, 1937). Local application to the bill of the sparrow is followed by a response when a total of 1.0 μg. of either substance is given (Pfeiffer, Hooker and Kirschbaum, 1944). The comb of the capon has responded to a total dose of 1.2 μg. (Emmens, 1939) and 0.2 μg. (Koch and Johnston, 1941) of androsterone when applied topically. The most sensitive tests for estrogens thus far proposed are those of Hartman and Littrell (1945), who report that the vagina of the immature rat opens in response to 0.02 cc. of blood from women in the middle of the cycle, and of Hartman, Littrell, and Tom (1946), who state that as little as 0.0004 μg. estradiol dipropionate will open the vagina of the weanling guinea pig. A lower sensitivity, however, was found by Lloyd, Rogers, and Williams (1946) for the rat test. Thus it seems that despite its low activity as assessed by systemic administration, progesterone can be detected in smaller amounts than the androgens and estrogens, although on a weight basis both possess relatively greater activity when administered systemically.

Despite the sensitivity and apparent specificity of the intra-uterine test for progesterone in the mouse, it has disadvantages at present that will seriously restrict its usefulness. Perhaps the greatest of these are the time and labor required. After making intra-uterine injections in over 200 mice, the total time needed for the injection of both uterine horns is rarely less than 15 minutes. Injecting 20 mice thus becomes a full and fatiguing day's work. Although the intensity of the response increases as the amount of injected progesterone increases, it has not thus far been possible to grade the response. The result is that the reaction has had to be treated on an all-or-none

basis that necessitates the identification of the minimal effective amount in a series of dilutions of the same material. A first approximation is possible if only one uterine cornu is injected: in this situation a bilateral response indicates that more than 0.004 μg. of progesterone has been given; if the response if given only by the treated segment, the amount injected is not less than 0.0002 μg. nor more than 0.005 μg. of progesterone.

A third disadvantage of the method is the care needed to assess the sections of the test segment. It is essential that the observer differentiate the stromal nuclei from the glandular nuclei. It is also essential that he be familiar with the appearance of the stromal nuclei in the untreated, ovariectomized mouse and in an endometrium exhibiting a response to an effective amount of progesterone. It has proved helpful, and sometimes necessary, for the observer to refresh his memory by reviewing known preparations of both types immediately before attempting to evaluate a test preparation. When this precaution is taken the identification of a positive response or the lack of response is not difficult.

Although the test is given by various crude materials, it will be apparent that assays of blood levels in various physiologic conditions would be premature until it is learned whether progestin in blood is free or "bound" as are the estrogens (Rakoff, Paschkis, and Cantarow, 1943; Roberts and Szego, 1946; Szego and Roberts, 1946) and until the proportions of such fractions are determined.

The relation of estrogen to the response to progesterone in the mouse appears to be quite different from that in the rabbit. Preliminary action of estrogen is not necessary as in the rabbit (Allen, 1930; McPhail, 1934), and the response does not seem to be enhanced by administration of estrogen. Moreover, no indication of antagonism between estrogen and progesterone, the significance of which in the rabbit has been stressed by Courrier (1930 et seq.), has been encountered. Apparently the responses to estrogen and progesterone are quite independent and may be elicited separately or concurrently when these substances are given systemically (Hooker, 1945) or locally.

The requirement of 16 days for regression of the stromal nuclei to the full condition of castration was unexpected. It is unknown whether the blood level of progesterone falls more slowly after ovariectomy than has been suspected or whether the morphological change in the nucleus is a slow process. It is interesting, however, that the castration change can be repaired within six hours.

Another point of interest is the failure of comparatively large doses of desoxycorticosterone acetate and testosterone to mimic the effect of progesterone when placed directly within the uterus. The former substance was luteoid by the nuclear test in some mice when given systemically (Hooker, 1945), and in the rabbit has been rated as being

one-tenth (Selye and Masson, 1943) to one-third (Van Heuverswyn, Collins, Williams, and Gardner, 1939) as active as progesterone when given systemically. Intra-uterine administration of an amount 30,000 times that of the effective amount of progesterone failed to mimic the action of progesterone in the mouse. Similarly, testosterone is considered to be 1/140–1/200 (Selye and Masson, 1943), or perhaps more (Klein and Parkes, 1937), as active as progesterone in provoking progestational changes in the rabbit when given systemically. However, an amount of testosterone 2400 times the effective dose of progesterone did not mimic the action of progesterone when injected directly into the uterus of the mouse. These observations again raise the question whether these substances are in themselves luteoid or manifest this action only after conversion of a small portion of the systemically administered quantity into another substance, possibly progesterone.

The finding of progesterone (or of a substance exhibiting the same action as that of progesterone) in ovarian follicular fluid is of interest in relation to the more or less indirect evidence accumulated for the production of progestin by the pre-ovulatory follicle (reviewed by Young, 1941). Apparently the only previous examination of follicular fluid for progestin was that of Duyvene de Wit (1938), who used the bitterling test. Although the test is not specific for progestin, this author reported activity corresponding to 8 μg. progesterone in 1 cc. of follicular fluid from swine and 5 μg. in 1 cc. of follicular fluid from cattle. These values are of the same order of magnitude as the concentrations suggested by a single test using the mouse.

SUMMARY

The assay consists of the injection, by means of micrometer bolt action on a 0.5 cc. syringe, of 0.0006 cc. of test material into a 5 mm. ligated segment of uterine horn in an adult CHI mouse ovariectomized 16 days earlier. The animal is autopsied 48 hours after injection, and the uterine segment is sectioned.

Under these conditions the minimal effective dose of progesterone in oil is 0.0002 μg. The criterion for a positive response is the transformation of the fusiform, dense stromal nuclei with clumped chromatin and no visible nucleoli, as seen in the endometrium of an untreated castrated animal, into plump, oval nuclei with conspicuous nucleoli and fine, evenly dispersed chromatin particles.

Estradiol, estrone, desoxycorticosterone acetate, and testosterone did not duplicate the action of progesterone on the stromal nuclei. The test was, however, positive for a crude extract of sows' corpora lutea, for plasma from pseudopregnant rabbits, for serum from ovariectomized mice injected with progesterone, and for liquor folliculi from sows' ovaries. Concomitantly administered estrogens neither interfered with nor enhanced the action of progesterone.

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THE RELATIONSHIP OF DIET TO THE EFFECT OF ADRENOCORTICOTROPHIC HORMONE UPON URINARY NITROGEN, GLUCOSE AND ELECTROLYTES

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IN A previous study (Ingle, Li and Evans, 1946) it was shown that the administration of pure adrenocorticotrophic hormone (ACTH) to normal rats force-fed a high carbohydrate diet caused hyperglycemia and glycosuria with an accompanying increase in the excretion of nitrogen and potassium and inhibition of growth. In the present experiments a comparison was made of the metabolic responses of normal rats to ACTH as related to the type of diet. During the administration of ACTH some of the animals on the high carbohydrate diet developed glycosuria. All of the animals showed inhibition of weight gains and an increase in urinary nitrogen, the most marked changes occurring in the high fat series and the least marked changes in the high protein series. The accompanying rise in urinary potassium was not clearly related to the nature of the diet. There were small, temporary increases in the excretion of sodium and chloride which may have been insignificant.

METHODS

Male rats of the Sprague-Dawley strain were maintained on a diet of Purina Dog Chow until they reached a weight of approximately 300 gm. They were then placed in metabolism cages and maintained on a fluid diet administered by stomach tube each morning (8:30 to 9:15 A.M.) and afternoon (4:15 to 5:00 P.M.). The technique of forced feeding and the diets used were modifications of those described by Reinecke, Ball and Samuels (1939). The diets were made according to Table 1. During the period of adaptation to forced feeding the amount of diet was increased gradually to prevent the development of "food-shock." The animals were brought to a full feeding on the fifth day. Amounts of 26 cc. per day of the high carbohydrate and of the high fat diets and 40 cc. per day of the high protein diet were given. These volumes of diet were estimated to be isocaloric and con-

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tained the same basal constituents. The sodium, potassium and chloride content of the high protein diet was higher than for the other two diets since the egg albumin and lactalbumin contained substantial amounts of these elements.

The animals were housed in an air-conditioned room in which the temperature was maintained at 74 to 78 degrees F. and the humidity at 30 to 35 percent of saturation. Twenty-four-hour samples of urine were collected at the same hour each day and were preserved with thymol and 1 gram of citric acid to insure the acidity of the samples for nitrogen analysis. The following methods of analysis were used: urinary glucose, Benedict (1911); urinary chloride, the modified Volhard-Harvey titration as described by Peters and Van Slyke (1932); and urinary sodium and potassium by the

TABLE 1. COMPOSITION OF FLUID DIETS. GRAMS

Constituent	High COH	High fat	High protein
Cellu flour (Chicago Dietetic Supply)	120	120	60
Osborne & Mendel Salt mixture	40	40	20
Wheat germ oil	10	10	5
Mazola oil plus 100 mgm. Vitamin K	10	10	10
Cod liver oil	10	10	5
Dried yeast (Pabst)	100	100	50
Starch (soluble-Difco)	330		
Dextrin	335		
Sucrose	335		
Egg albumin (Merck)	160	160	200
Lactalbumin			200
Casein (Labco)			50
Mazola oil		200	65
Butter fat		260	
Water to make total volume of	2000 cc.	2000 cc.	1500 cc.

flame photometer, Barnes, Richardson, Berry and Hood (1945). In order to insure the reliability of this method it was necessary to test the standards at frequent intervals during the operation of the instrument instead of depending upon a single standard curve. Optimal accuracy is obtained by the procedure of matching the response of standard solutions and the test solutions. The determination of non-protein nitrogen was by the micro-Kjeldahl procedure as follows: proteins were precipitated as salts of tungstic acid by the Folin-Wu procedure; the organic matter was oxidized by sulfuric acid and hydrogen peroxide; the ammonia was distilled off into a standard acid solution and titrated with a standard base.

The ACTH was prepared by the procedure of Li, Evans and Simpson (1943). Following control periods of 14 days, the animals were given ACTH by subcutaneous injection in divided doses every 2 hours for a total of 8 injections per day.

EXPERIMENTS AND RESULTS

Experimental. In each of the three experiments all of the animals were observed during a control period of 14 days. Analyses were run for 7 days prior to the administration of ACTH. In experiment 1, two rats on each of the three diets were injected with 1 mgm. of ACTH per day for 21 days. An equal number of control animals were studied.

Experiment 2 was identical with experiment 1 except that the dosage of ACTH was 3 mgm. per day. In Experiment 3, 8 mgm. of ACTH per day was injected for 10 days and 4 rats given ACTH were maintained on high carbohydrate diet. The other details remained the same. At the end of the injection period all of the animals were killed for necropsy and the tissues were submitted to Dr. B. L. Baker, Department of Anatomy, University of Michigan School of Medicine for histochemical study. Dr. Baker's findings will be reported separately.

Urinary glucose. None of the rats on the high fat and high protein

TABLE 2. THE RELATIONSHIP OF DIET TO THE EFFECT OF ACTH UPON URINARY NON-PROTEIN NITROGEN IN THE NORMAL RAT

ACTH mgm. day	Days treatment	Average* daily increase above values of pre-injection period, mgm.			Average* daily increase above values obtained at same time on control rats, mgm.		
		COH	Fat	Protein	COH	Fat	Protein
1	21	54	67	26	44	87	17
3	21	61	77	30	53	88	18
8	10	78	95	64	71	85	42

* Two control and 2 experimental animals were represented at each dosage level for each diet with the exception that 4 rats were given 8 mgm. daily of ACTH on the high carbohydrate diet.

diets developed glycosuria during the administration of ACTH. On the high carbohydrate diet each of the two rats given 1 mgm. of ACTH daily developed glycosuria, the 2 given 3 mgm. daily did not, and 3 of the 4 rats given 8 mgm. daily developed glycosuria. The data are summarized in Table 1.

Urinary non-protein nitrogen. All of the rats given ACTH showed an increase in the excretion of non-protein nitrogen. The extent of the rise was related to the size of dose. The animals on the high protein diet showed the smallest response and the animals on the high fat diet the greatest response. The relative changes from control values were the same when they were compared either to values of the pre-injection period for the same animals or to the values for control animals studied at the same time. The results are recorded as averages in Table 2.

Body-weight. All of the rats gained weight during the control periods but ACTH inhibited growth. The changes in weight paralleled the changes in nitrogen balance. The animals on the high protein diet showed the smallest response and those on the high fat diet the greatest response. With 1 mgm. of ACTH daily the rats on the high protein diet continued to gain weight but at a slower rate. All of the remaining animals lost weight during administration of the hormone in amounts that were proportional to the dose. The data are summarized as averages in Table 3.

Urinary potassium. The administration of ACTH caused an in-

crease in potassium loss but there was no clear relationship between the extent of response and the nature of the diet. The data are summarized in Table 4.

Urinary sodium and chloride. When averages for all of the animals were compared without reference to the dose of ACTH or the diet there was found a small and statistically unreliable increase in the

TABLE 3. THE RELATIONSHIP OF DIET TO THE EFFECT OF ACTH UPON CHANGE IN WEIGHT. Grams

ACTH mgm. day	Days treatment	Average* change in weight during the injection period			Average* difference in weight between controls and experimental at end of injection period		
		COH	Fat	Protein	COH	Fat	Protein
1	21	−10	−17	24	46	61	30
3	21	−28	−33	− 8	59	66	53
8	10	−25	−25	−17	34	39	30

* Two control and 2 experimental animals were represented at each dosage level for each diet with the exception that 4 rats were given 8 mgm. daily of ACTH on the high carbohydrate diet.

TABLE 4. THE RELATIONSHIP OF DIET TO THE EFFECT OF ACTH UPON URINARY POTASSIUM IN THE NORMAL RAT

ACTH mgm. day	Days treatment	Average* daily increase above values of pre-injection period, mgm.			Average* daily increase above values obtained at same time on control rats, mgm.		
		COH	Fat	Protein	COH	Fat	Protein
1	21	9.3	12.4	14.1	4.1	10.0	3.1
3	21	10.0	4.4	6.1	5.9	11.5	13.3
8	10	20.4	22.2	21.9	17.4	16.7	15.5

* Two control and 2 experimental animals were represented at each dosage leve with the exception that 4 rats were given 8 mgm. daily of ACTH on the high carbohydrate diet.

loss of these ions immediately following the beginning of ACTH injections. The average increase in loss of chlorides was 7.6 and 4.2 mgm. for the 1st and 2nd days respectively and an average increase in loss of sodium of 5.2 and 5.0 mgm. for the 1st and 2nd days respectively following the administration of ACTH.

Necropsy. The weights of single adrenal glands are recorded in Table 5. Each contralateral gland was preserved for histologic study. ACTH caused a marked cortical hypertrophy. In this small series of rats there was no significant relationship between the weights of adrenal glands and the nature of the diet. All of the animals given ACTH showed almost complete atrophy of the thymus. In each animal treated with ACTH there was some regression in size of the testes and a marked regression in the size of the seminal vesicles. No other gross pathological changes were noted.

DISCUSSION

The general effects of ACTH observed in these experiments are identical with those observed by Ingle, Li and Evans (1946). It was expected that the forced feeding of the high carbohydrate diet would favor the production of glycosuria by ACTH. However, the results on nitrogen balance and body-weight were contrary to the hypothesis of one of us (D. J. I.) that ACTH should have its greatest effect upon nitrogen balance in animals fed a high protein diet. Although the numbers of animals were small the consistency of the results obtained under conditions of rigid control of dietary intake makes it probable

TABLE 5. ADRENAL WEIGHTS IN RELATIONSHIP TO DIET AND TO THE ADMINISTRATION OF ACTH

ACTH mgm. day	Days treatment	Weights of single adrenal glands					
		High carbohydrate		High fat		High protein	
		ACTH	Control	ACTH	Control	ACTH	Control
1	21	63	32	66	24	65	27
		57	21	57	32	51	23
3	21	62	20	70	24	60	21
		74	18	62	24	73	24
8	10	114	22	54	20	56	25
		58	20	38	24	72	24
		80					
		46					

that the observed differences are valid. At the present time, a satisfactory explanation is lacking. It should be of interest to study the metabolic effects of adrenal cortical extracts and steroids in relation to diet. The increase in urinary potassium during the administration of ACTH may be related to the increased breakdown of the tissues which characterizes the early stages of hyperadrenocortinism but there was no apparent relationship between the extent of nitrogen loss and of potassium loss.

In none of our experiments with pure ACTH have we observed a retention of sodium and chloride. Does ACTH fail to stimulate the secretion of those adrenal cortical steroids which cause retention of sodium and chloride? The important cytochemical studies of Greep and Deane (1947) are relative to this problem. The C-11, 17-oxygenated compounds have the property of causing a sharp temporary increase in the excretion of these ions. Some rise in the excretion of sodium and of chloride was observed during the first 2 days of ACTH administration but the change was small and may have been owing to chance. If ACTH stimulates the secretion of both the "sodium-retaining" and the "sodium-excreting" hormones simultaneously the

effect of each may be masked. Mason and co-workers (1947) found no effect of pure ACTH upon the sodium balance of a normal young woman. To the contrary, Thorn and co-workers (1947) have observed a striking sodium-retaining effect of a partially purified preparation of ACTH in a man having hypopituitarism. The effect of adrenal cortical insufficiency upon electrolyte balance has been well studied as have the properties of one steroid compound, 11-desoxycorticosterone. Relatively little is known of the effects of the other biologically active adrenal cortical steroids or of the function of intact adrenal cortices and the anterior pituitary in relation to the distribution and excretion of inorganic ions. It is clear that a change in the functional activity of the adrenal cortex is not the only factor regulating electrolyte metabolism, for physiologically economical adaptations in sodium, chloride and potassium balance can be demonstrated in adrenalectomized rats maintained on a steady intake of adrenal cortical extract (Ingle, unpublished).

SUMMARY

Normal male rats were force-fed high carbohydrate, high fat and high protein diets in isocaloric amounts. Pure ACTH was given in 8 divided doses of 1, 3 and 8 mgm. daily for 21, 21 and 10 days respectively. The effects of the hormone were studied in relation to diet. During the administration of ACTH some of the rats on the high carbohydrate diet developed glycosuria. All of the animals excreted more non-protein nitrogen during treatment with ACTH, the most marked changes occurring in the high fat series and the least marked in the high protein series. ACTH inhibited growth and caused a loss of weight in most of the animals. The rats on the high protein diet showed the smallest response and the rats on the high fat diet the greatest response. All of the animals excreted more potassium during the administration of ACTH but there was no clear relationship between the extent of response and diet. The changes in sodium and chloride balance were small and irregular. Immediately following the beginning of ACTH injections there was a small temporary increase in sodium and chloride loss which may have been owing to chance. There was a striking hypertrophy of the adrenal glands and almost complete atrophy of the thymus. The extent of response was not shown to be related to the type of diet.

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GONADOTROPHIC HORMONE:* PROTECTION AGAINST INACTIVATION

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GONADOTROPHIC hormones stimulate gonadal growth, maturation and secretion. The simultaneous administration of certain substances modifies the action of gonadotrophins. These substances have been classified according to their mode of action, as (1) synergists and (2) augmentors.

Synergists are themselves gonadotrophic substances which exert their modifying influence at the end-organ—the gonad. Synergism occurs whether the synergist is injected at the same site, or at a site distant from the gonadotrophin injection. It is exemplified by the increase in ovarian response which results from the simultaneous administration of chorionic gonadotrophin and pituitary gonadotrophin (Leonard, 1932).

Augmentors exert their modifying influence by delaying absorption of gonadotrophins from the site of injection (Evans, Hines, Ceithaml and Koch, 1940). They are inert if injected at a site different than the gonadotrophin (Maxwell, 1934; Bischoff, 1945). They include such chemically diverse materials as whole blood (Casida, 1936), aluminum hydroxide (McShan and Meyer, 1945) and egg white (Hellbaum, 1936).

It is the purpose of this communication to demonstrate a third type of gonadotrophin enhancing mechanism which operates *in vitro* in contrast to augmentors and synergists which exert their action *in vivo*. The mechanism involves protecting against the *in vitro* inactivation of gonadotrophins by certain materials named "protectors."

MATERIALS AND METHODS

Twenty-two to twenty-four day-old Sprague-Dawley female rats were used to assay the gonadotrophins. One cc. of the materials to be assayed was injected twice daily for 3 days. The animals were killed 24 hours after the last injection. Ovarian and uterine weights were taken as the assay endpoints.

Gonadotrophins from four sources were investigated. (1) Adult male and female rat anterior pituitary glands served as the source of gonadotrophic hormone in the majority of experiments. The donor rats were castrated 20

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to 58 days prior to removal of the glands in order to increase gonadotrophin content. After decapitation, the anterior lobes were removed immediately, macerated by alternately drawing them into and expelling them from a syringe, and suspended in sufficient Locke's solution so that each 6 cc. (amount for one assay rat) contained the equivalent of 10 mg. of 20-day castrate pituitary tissue. (2) Lyophilized sheep anterior pituitary powder* was dissolved in Locke's solution so that each 6 cc. contained 0.25 mg. (3) Chorionic gonadotrophin powder was dissolved in Locke's solution so that each 6 cc. contained 10 mg. (4) Human male castrate urinary gonadotrophin, concentrated by the alcohol precipitation method of Heller and Chandler (1942), was dissolved in Locke's solution so that each 6 cc. contained the hormone concentrated from a two-hour urine aliquot.

Procedures were conducted immediately after the solutions of gonadotrophins were prepared. These consisted of incubation at 37°C., shaking at 37°C., shaking at 3°C., and storage at 3°C. with and without the addition of one of the following substances: whole blood, testis slices, brain slices, egg white, aluminum hydroxide, prepuberal urine and ultrafilter concentrate of prepuberal urine. All materials were stored at 3°C. during the three-day injection period. Solutions were shaken in 125 cc. Erlenmeyer flasks in a Kahn bacteriological shaker.

Whole blood was obtained from adult rats by intracardial puncture. Sufficient blood was added so that each 6 cc. of solution contained 0.33 cc. of whole blood. Rat testis slices were added to the solution so that each 6 cc. contained 500 mg. of sliced testis. Rat brain slices were likewise added so that each 6 cc. of solution contained 500 mg. of sliced brain. Egg white was added so that 6 cc. of solution contained 0.33 cc. egg white. Aluminum hydroxide, prepared according to Johnson and Bradley quoted by McShan and Meyer (1945), was added so that each 6 cc. solution contained 100 mg. of aluminum hydroxide. In the experiment where prepuberal urine was used, the rat anterior pituitary glands were taken up in urine, instead of Locke's solution, so that each 6 cc. of urine contained 10 mg. rat anterior pituitary. In the experiment where ultrafilter concentrate of prepuberal urine was used, sufficient material was added so that each 6 cc. of solution contained the material concentrated from a 4-hour urine aliquot.

RESULTS

Inactivation of castrated rat pituitary gonadotrophin suspension. Control pituitary suspensions were kept at 3°C. for comparison with suspensions that were incubated at 37°C. or shaken at 3° and 37°C. The data of Table 1 show that shaking for 2.5 hours at 3°C. and incubation for 2.5 hours at 37°C. caused little if any inactivation of the gonadtrophin whereas shaking for 2.5 hours at 37°C. caused complete inactivation. Reference to Table 2 offers additional evidence that shaking at 37°C. for 2.5 hours completely inactivates rat pituitary gonadotrophin. A total of five separate comparisons were made. Whereas suspensions stored at 3°C. showed demonstrable gonadotrophic potency in each assay, suspensions shaken at 37°C. for 2.5

* Generously supplied by Mr. Frederic Fenger of Armour and Company.

hours elicited average ovarian weights ranging from 8.2 mg. to 13.4 mg. and no uterine stimulation in any assay. This may be compared to the uninjected control average ovarian weight of 10 mg. and no uterine stimulation. Thus it may be concluded that rat pituitary gonadotrophin is totally inactivated by shaking at 37°C. for 2.5 hours. (In all instances where average ovarian weights fell within the range of uninjected controls, no uterine stimulation was noted. In all instances where ovarian weights greater than the range of uninjected

TABLE 1. EFFECT OF SHAKING AND TEMPERATURE UPON INACTIVATION OF RAT PITUITARY GONADOTROPHIN

	Rat gonadotrophin 3° C.	Rat gonadotrophin shaken 2.5 hours at 3° C.	Rat gonadotrophin 37° C. for 2.5 hours	Rat gonadotrophin shaken 2.5 hours at 37° C.	No. of assay rats per procedure
Average ovarian weights, mgs.	34.2	27.8	29.5	11.7	8

Average ovarian weight of 40 uninjected controls = 10.0 mg. range: 4.8 to 16.5. (This value was obtained from uninjected controls included in each series of assays conducted and applies to Tables 2, 3, 4 and 5 also.)

TABLE 2. PROTECTION AGAINST INACTIVATION OF RAT PITUITARY GONADOTROPHIN

Protector (Amount per 6 cc.)	Rat gonadotrophin 3° C. No protector	Rat gonadotrophin shaken 2.5 hours at 37° C. No protector	Rat gonadotrophin + protector shaken 2.5 hours at 37° C.	Rat gonadotrophin shaken 2.5 hours at 37° C. then add protector	Rat gonadotrophin + protector 3° C.	No. of assay rats per procedure
.33 cc. whole blood	46.1	10.3	44.4	8.5	66.3	6
500 Gms. sliced testis	54.5	11.7	38.7	8.5*	—	6
500 Gms. sliced brain	53.6	13.4	29.6	—	—	3
.33 cc. egg white	36.8*	10.6*	20.3	10.1	48.8	3
100 mg. $Al(OH)_3$			30.0	10.2	59.2	2
6 cc. prepuberal urine	27.2	8.2	6.6	7.5	31.1	3
4 hour ultra filter concentrate of prepuberal urine			8.1	—	—	3

* 3 rats per dose.
Figures represent average ovarian weights of assay rats expressed in milligrams.

controls were encountered, maximal uterine stimulation was noted. This generalization applies to Tables 1 through 5.)

Protection against inactivation of castrated rat pituitary suspensions was afforded by the addition of whole blood, rat testis slices, rat brain slices, egg white and aluminum hydroxide to the rat gonadotrophin before shaking at 37°C. The data of Table 2 show that the addition of whole blood afforded the best protection against inactivation, whereas testis and brain slices, egg white and aluminum hydroxide afforded less complete protection. Raw prepuberal urine

and concentrates of such urine prepared by ultrafiltration offered no protection. None of the above materials, using the same amount of material per assay animal as added to 6 cc. of gonadotrophin, had any demonstrable gonadotrophic activity.

An alternate explanation for the detection of gonadotrophins in the suspensions after shaking at 37°C. in the presence of a foreign material is that the added material served as an augmentor. Thus inactivation might occur as usual but sufficient augmentation might take place to restore gonadotrophic potency. Reference to Table 2 indicates that this possibility was ruled out for the action of whole

TABLE 3. PROTECTION AGAINST INACTIVATION OF RAT PITUITARY GONADOTROPHIN DURING 3 DAYS STORAGE AT 3° C.

Protector (Amount per 6 cc.)	Rat gonadotrophin 3° C. No protector	Rat gonadotrophin mixed with protector during 3-day injection period	Rat gonadotrophin + protector added at each injection	No. of assay rats per procedure
.33 cc. blood	34.2	48.3	32.5	8

Figures represent average ovarian weights of assay rats expressed in milligrams

blood, testis slices, egg white and aluminum hydroxide by adding these substances after shaking at 37°C. had been completed. In no instance was gonadotrophic activity noted.

Adding whole blood, egg white and aluminum hydroxide to the control pituitary gonadotrophin suspensions kept at 3°C. resulted in an increase in gonadotrophic activity. This could be due to augmentation, synergism (assuming that the blood contains some gonadotrophic hormone), or if inactivation occurs at 3°C. over a 3-day period, it could be due to protection against such inactivation.

Inactivation at 3°C. for three days. The data of Table 3 show that addition of whole blood to a suspension of rat pituitary gonadotrophin cause an increased gonadotrophic response. If this increased response were due to augmentation or synergism, then the same order of response should be elicited whether the blood was in contact with the gonadotrophin for the 3-day injection period or in contact only at the time of injection. Therefore blood was added to an aliquot of the the suspension at each injection. No increase in potency was noted (Table 3). It is therefore concluded that (1), inactivation occurs in 3 days at 3°C. and that (2), whole blood exerts its enhancing effect by protecting against inactivation and not by augmentation or synergism.

Protection against inactivation of sheep gonadotrophin. Sheep gonadotrophin was inactivated by shaking for 2.5 hours at 37°C. Inactivation was prevented by adding aluminum hydroxide before incubation but not be adding aluminum hydroxide after incubation (Table 4).

Shaking for 2.5 hours at 37°C. failed to inactivate human urinary and chorionic gonadotrophin (Table 5). Even shaking for 5 hours at 37°C. failed to decrease the gonadotrophic potency of urinary extracts. Since both extracts are relatively crude preparations, it may be that the original material contains a substance which already

TABLE 4 PROTECTION AGAINST INACTIVATION OF SHEEP PITUITARY GONADOTROPHINS

Protector (Amount per 6 cc.)	Sheep gonadotrophin No protector	Sheep gonadotrophin shaken 2.5 hours at 37° C. No protector	Sheep gonadotrophin + protector shaken 2.5 hours at 37° C.	Sheep gonadotrophin shaken 2.5 hours 37° C then add protector	Sheep gonadotrophin + protector 3° C.	No. of assay rats per procedure
100 mg. $Al(OH)_3$	46.1	9.4	44.1	18.2	49.0	3

Figures represent average ovarian weights of assay rats expressed in milligrams.

TABLE 5. FAILURE OF SHAKING AT 37° C. TO INACTIVATE HUMAN URINARY AND CHORIONIC GONADOTROPHINS

Type of gonadotrophin	Gonadotrophin 3° C.	Gonadotrophin shaken 2.5 hours at 37° C.	Gonadotrophin shaken 5 hours at 37° C.	No. assay rats per procedure
Human urinary gonadotrophin	45.7	44.8	40.2	3
Chorionic gonadotrophin	23.8	22.3	—	3

Figures represent average ovarian weights of assay rats expressed in milligrams.

serves as a protector. However, a more plausible explanation, considering that we were unable to demonstrate any "protector" in urine or urine concentrate, is that these gonadotrophic hormones are more stable than gonadotrophins extracted from pituitary glands.

DISCUSSION

Inactivation. Pituitary gonadotrophic hormones, although comparatively stable in dry powder form, are notoriously unstable in aqueous solution. Rowlands (1935) found no loss of potency when pituitary gonadotrophin *powder* was heated to 100° C. for one hour. However, heating an aqueous solution of hormone just to boiling caused complete loss of potency (Van Dyke and Wallen-Lawrence, 1933). Solutions stored at room temperature for 9 days lost approximately 50% of their original potency (Rowlands, 1935). In contrast, storage of sheep pituitary extracts at icebox temperatures for periods of months resulted in relatively little loss of gonadotrophic potency as compared with solutions that were stored for 4 days at icebox temperatures (Rowlands, 1935; Van Dyke and Wallen-Lawrence, 1933).

The methods of assay of gonadotrophic substances in general use involve a 3 to 5 day injection period during which the solutions to be

assayed are stored at icebox temperature. We have been unable to find any previous report concerning the degree of inactivation that occurs during this 3 to 5 day period of storage at icebox temperature. In the present study, partial inactivation of rat gonadotrophin during 3 days storage at 3°C. has been demonstrated by the addition of a substance (whole blood) capable of protecting the hormone from inactivation (Table 3). On the other hand, Rowlands (1935) and Van Dyke and Wallen-Lawrence (1933) observed relatively little loss of potency of sheep pituitary extract stored at icebox temperatures for 5 to 12 months, as compared to solutions stored at icebox temperatures for 4 days. Two possible explanations of this apparent incongruity can be made. (1) Their sheep pituitary extracts were more stable than the rat pituitary suspension used in this study. (2) The inactivation of pituitary gonadotrophin solutions stored at icebox temperatures occurs, for the most part, during the first few days of storage.

Chorionic gonadotrophin is likewise much more stable in dry powder form than in aqueous solution. Although heating the dry powder at 100°C. for three hours results in no loss in potency, heating an aqueous solution to 100°C. for five minutes results in almost complete inactivation (Askew and Parkes, 1933). Although unstable in the aqueous state, chorionic gonadotrophin solutions appear to be more stable than aqueous pituitary gonadotrophin preparations (D'Amour and D'Amour, 1938). The observation that chorionic gonadotrophin was not inactivated by shaking at 37°C. is in accord with past experience that chorionic gonadotrophins are more stable than pituitary gonadotrophins.

Although it is well known that proteins are denatured by shaking (Wu and Ling, 1927), the extreme susceptibility of pituitary gonadotrophic hormones to shaking at 37°C. has not, to our knowledge, been previously demonstrated. Denaturation by shaking involves the mechanism of surface denaturation, the function of shaking being to increase the formation of new surface. It is interesting to note that surface denaturation of urease results in loss (probably complete) of biological activity (Langmuir and Schaefer, 1939).

Synergism. Certain gonadotrophic hormones are capable of enhancing the effects of other gonadotrophins to a greater degree than can be explained by simply adding the effects of the two hormones when given separately. Leonard (1932) found that a combination of chorionic gonadotrophin and pituitary gonadotrophin increased the weight of the assay rats' ovaries more than double that which could be explained by simply adding the effects of the two materials when given separately. He further observed that this synergistic action occurred whether the two materials were combined and injected at the same site or if the materials were kept separate and injected at separate sites.

Synergism has also been demonstrated for the pituitary gonadotrophins, follicle-stimulating hormone (FSH) and interstitial-cell-stimulating hormone (ICSH, also known as luteinizing hormone, LH). Fevold, Hisaw and Leonard (1931) found that regardless of dosage, a purified preparation of ICSH was incapable of increasing the ovarian weights of immature female rats. However, when combined with FSH, ICSH was capable of enhancing the ovarian weight increasing action of FSH as much as five-fold. Furthermore, this synergistic action occurred if ICSH was administered alone for three days immediately followed by the injection of FSH for three days. This effect was also demonstrated using hypophysectomized assay animals. They concluded that the synergistic effect of ICSH was a direct action on the ovary.

Augmentation. As mentioned beforehand, numerous materials that alone have no demonstrable gonadotrophic action are capable of augmenting the action of certain pituitary gonadotrophin preparations. In view of the great number of known "augmentors" it would appear highly improbable that each augmented by a different mechanism. Available evidence indicates that augmentation is, in each instance, due to delayed absorption from the site of injection. The end result is to bring about a more gradual release of hormone from the site of injection, thus simulating the probable manner in which the pituitary secretes the gonadotrophic hormone into the blood stream. The explanation of augmentation on such a basis rests on the assumption that a more gradual release of hormone into the blood stream *is* more effective in stimulating the gonads. It is therefore interesting to note that Meyer and McShan (1941) found beef and sheep anterior pituitary gonadotrophic potency to be enhanced approximately ten-fold by increasing the number of injections from 2 to 24 daily.

Augmentation does not occur if augmentor and hormone are injected at different sites (Maxwell, 1934; Bischoff, 1945). This is in accord with the view that augmentation is due to delayed absorption from the site of injection. The possibility still remains, however, that augmentation is due to an *in vitro* reaction between augmentor and hormone. Evans, Ceithaml, Hines and Koch (1940) tested this possibility by mixing augmentor and hormone *in vitro* and injecting directly into the blood stream (intracardially). Augmentation did not occur when injections were made intracardially whereas it did occur when injections were made subcutaneously. They further showed that adrenalin injected along with the hormone caused an increase in gonadotrophic potency. It would appear, therefore, that the augmentation of pituitary gonadotrophic hormones by a variety of nonspecific materials can be adequately explained on the basis of delayed absorption of hormone from the site of injection.

Although augmentation can be adequately explained on the basis

of delayed absorption, this does not mean that in each instance the mechanism of delaying absorption is the same. Bischoff (1938) has demonstrated that zinc sulfate delays absorption by precipitating the gonadotrophic hormones. Merthiolate augments by causing a local reaction at the site of injection (Chen and Van Dyke, 1938). Tannic acid retards absorption by forming an insoluble tannate of the gonadotrophic hormone (Bischoff, 1936). Sodium lauryl sulfate produces an edematous reaction thus forming a depot of hormone containing fluid which is slowly resorbed (Bischoff, 1945).

Augmentors may be classed as "strong" or as "weak" according the degree of augmentation they produce. The following materials have been shown to be capable of causing a three to ten-fold increase in gonadotrophic potency and thus may be termed "strong" augmentors: aluminum, zinc, magnesium and ferric hydroxides (McShan and Meyer, 1945); zinc sulfate (Maxwell, 1934); yeast extract, yeast ash and copper salts (Fevold, Hisaw and Greep, 1936); male urine extract, milk, egg white, lemon, horse thyroid and beef liver (Hellbaum, 1936); whole blood, red blood cells, hemoglobin, hemin and heme (McShan and Meyer, 1937); sodium lauryl sulfate (Bischoff, 1945); tannic acid (Bischoff, 1936); and merthiolate (Chen and Van Dyke, 1938). "Weak" augmentors, which cause a barely detectable to two-fold increase in gonadotrophic potency, include the following substances: casein (Saunders and Cole, 1936); serum (Cole and Hart, 1934); plasma (Casida, 1936); leukocytes (Kraatz, 1936) and chlorophyll (Breneman, 1939).

The assay methods used in demonstrating augmentation have usually involved a three to five day injection period. Enhanced gonadotrophic potency has been noted if the material in question has been mixed with the hormone during the injection period but not if the materials have been kept separated and injected at separate sites. Thus, the material added could be acting as an augmentor, as a protector or as both, It is not probable, considering the degree of increase produced, that protection can fully explain the gonadotrophin enhancing action of strong, "augmentors." It is possible, however, that protection against inactivation accounts for *part* of the increased gonadotrophic potency. In the case of "weak augmentors" it is possible that protection is *the* mechanism involved.

Separation of the three gonadotrophin enhancing mechanisms. By definition, synergism is an *in vivo* mechanism whose locus of action is the gonad, augmentation is an *in vivo* mechanism whose locus of action is the site of injection, and protection is an *in vitro* mechanism. This is the basis for identifying the mechanism involved when an increase in potency is noted upon the addition of a given substance to a gonadotrophic hormone solution. Identification of the mechanism involved can be accomplished by the following three procedures:

1. Injection of the substance in question and gonadotrophin at different sites, the materials being kept separate while *in vitro*.

2. Addition of the substance in question to the gonadotrophin just prior to each injection.

3. Addition of the substance in question to be known gonadotrophin during the entire 3 to 5 day period of injection.

The mechanism involved is identified as follows:

a. If an increase is noted with procedures 1, 2 and 3—the mechanism involved is synergism.

b. If an increase is noted only with procedures 2 and 3, the mechanism involved is augmentation.

c. If an increase is noted only with procedure 3, the mechanism involved is *protection* against inactivation.

SUMMARY AND CONCLUSIONS

To the two known gonadotrophin enhancing mechanisms, augmentation and synergism, can be added a third—protection against inactivation.

Rat and sheep gonadotrophins are completely inactivated by shaking at 37°C. for 2.5 hours. Rat gonadotrophin is only slightly inactivated by incubation at 37°C. for 2.5 hours, or by shaking for 2.5 hours at 3°C.

Addition of whole blood, egg white, testis slices, brain slices or aluminum hydroxide to rat pituitary gonadotrophin offers protection against inactivation from shaking at 37°C. for 2.5 hours.

Addition of aluminum hydroxide protects sheep pituitary gonadotrophin against inactivation from shaking at 37°C. for 2.5 hours.

Storage at 3°C. for 3 days slightly inactivates rat pituitary gonadotrophins. Addition of whole blood protects against such inactivation.

Protection against *in vitro* inactivation must be considered in concluding that a substance acts as an augmentor, especially since some of the known augmentors such as whole blood, egg white and aluminum hydroxide are also protectors.

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EFFECT OF GONADOTROPHINS ON THE CHOLESTEROL CONTENT OF THE TESTES OF IMMATURE RATS*

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THERE are two possible mechanisms whereby the gonadotrophic hormones of the pituitary and chorion might affect the activity of the androgen-producing cells of the male gonad: (a) by acting primarily upon the vascular system of the target organ, (b) by stimulating specific cell function or functions, possibly by virtue of a biocatalytic action, with increased vascularization of the organ secondary to increased metabolic activity of the cells. The identity of the specific intracellular reactions which may be affected by the gonadotrophic hormones is at present a matter of conjecture. It is the purpose of the present paper to suggest a possibly useful point of view for the analysis of the biochemical mechanism of action of the gonadotrophins.

In recent years Sayers and his colleagues (1944, 1946) have demonstrated that the administration of purified adrenocorticotrophic preparations to the rat results in a quick, initial depletion of the esterified fraction of cholesterol in the adrenal glands with an approximately concurrent reduction in the ascorbic acid content of the glands. Associated with these changes in the chemical composition of the adrenals, liver glycogen and urinary nitrogen analyses have revealed that adrenocorticotrophic hormone administration results in an increased output of the adrenocortical hormone which is functionally related to gluconeogenesis. Sayers and his co-workers (1946) have suggested that the adrenocorticotrophic hormone may function biocatalytically in the conversion of cholesterol to adrenocortical hormone.

There are certain evident similarities between the gonads and the adrenals. Each contains both free and esterified cholesterol, each is the site of production of at least one steroid hormone, and each is under trophic control which is mediated by an anatomically remotely arising protein or group of proteins. Therefore, it was postulated that

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the gonadotrophins participate in the cholesterol metabolism of the gonads in a manner analogous to that which has been described in connection with the hypophysio-adrenal axis. The following experiments were carried out to test this hypothesis.

MATERIALS AND METHODS

Healthy male albino rats (Albino Farms) reputed to be 21 days old were used in these experiments. They were fed a diet of Purina chow and allowed access to water *ad libitum*. Endocrine gland and empty seminal vesicle weights were obtained on a micro torsion balance. The gonadotrophic preparations used in this study were: (1) Follutein (Squibb), a commercial concentrate of human pregnancy urine. (2) Gonadaphysin (Searle),[1] a commercial concentrate of sheep anterior pituitary glands. The former preparation was found to have marked interstitial cell stimulating properties and will be referred to hereinafter, for convenience, as "ICSH." The latter preparation, while a potent follicle stimulating substance, was found to have a comparatively small effect on androgen production.[2] This preparation will hereinafter be referred to as "FSH." This code is not to be construed as implying that the authors regard these substances as purely interstitial-cell-stimulating or follicle-stimulating. All cholesterol analyses were done in duplicate by the method of Schoenheimer and Sperry as outlined in Macy (1942).

Differences between means were regarded as statistically significant when they exceeded thrice the standard error of the difference.

EXPERIMENTAL DESIGN AND RESULTS

In the first experiment twenty-four 21-day old animals were injected with a single dose of 50 units of "ICSH" subcutaneously. Twenty-four controls were similarly injected with physiological salt solution. At intervals of 6, 12, 24, and 48 hours groups of six "ICSH" animals and six controls were sacrificed, and testes weighed and analyzed for free and total cholesterol. The results are given in Table 1. It was found that there was a significant reduction in the per cent of total cholesterol at 24 hours after injection and a proportionate reduction in free cholesterol, but that these differences had disappeared by 48 hours. Determination of the water content of the glands of similarly prepared animals in a different experiment revealed that when the cholesterol per cents were calculated on a dry weight basis, there was no significant difference between the two groups of animals. Calculation of either free or esterified cholesterol in milligrams per 100 grams of rat revealed no significant difference between the "ICSH" animals and the controls.

In the next experiment 36 animals were treated with 20 units of the "ICSH" preparation twice daily for from one to six days. Control

[1] Obtained through the courtesy of Mr. Stuart Barlow of the Searle Company.

[2] Three different lots of Gonadophysin were found to vary appreciably in their ICSH activity as judged by the seminal vesicle weight assay method, yielding seminal vesicle weights of 22, 32, and 47 mg/100 gm. of rat respectively as opposed to normals of 13, 18, and 15 mg. per 100 gm. of rat.

animals were injected with appropriate amounts (0.1 ml.) of physiological saline. Six "ICSH" animals and six controls were sacrificed daily, seminal vesicles and testes weighed, and testes analyzed for free and total cholesterol. Results of this experiment are presented in

TABLE 1. EFFECT OF A SINGLE 50 UNIT SUBCUTANEOUS DOSE OF FOLLUTEIN ("ICSH") ON THE TOTAL CHOLESTEROL CONTENT OF THE TESTIS. MEANS AND STANDARD ERRORS FOR GROUPS OF SIX RATS ARE GIVEN (* SIGNIFIES 5 RATS PER GROUP)

Hrs. after injection	Treatment	Body wt.	Total cholesterol p.c. testis wet wt.	Tesis wt. mg./100 gm. rat
6	Saline	34 ± 2.5	0.31 ± 0.018	486 ± 36
6	"ICSH"	28 ± 2.2	0.31 ± 0.012	507 ± 31
12	Saline	31 ± 3.9	0.34 ± 0.024	486 ± 29
12	"ICSH"	34 ± 3.6	0.25 ± 0.006	648 ± 54
24	Saline	38 ± 3.9	0.29 ± 0.019	552 ± 13
24	"ICSH"*	35 ± 2.5	0.21 ± 0.008	689 ± 44
48	Saline	38 ± 2.4	0.27 ± 0.005	562 ± 37
48	"ICSH"	35 ± 2.4	0.27 ± 0.014	550 ± 29

TABLE 2. EFFECT OF "ICSH" ON TESTIS CHOLESTEROL CONTENT AND SEMINAL VESICLE WEIGHTS. MEANS AND STANDARD ERRORS FOR GROUPS OF 6 RATS ARE GIVEN. THE DOSE OF "ICSH" WAS 20 U. TWICE DAILY, SUBCUTANEOUSLY (* SIGNIFIES 5 RATS PER GROUP)

Days of injection	Treatment	Body wt.	Testis wt. mg./100 gm. of rat	Total cholesterol p.c. testis (wet wt.)	Total cholesterol mg./100 gm. of rat	Seminal vesicle wt. mg./100 gm. of rat
1	Saline	39 ± 3.1	491 ± 52	0.30 ± 0.018	1.43 ± 0.090	14.1 ± 1.4
1	"ICSH"	45 ± 2.0	681 ± 16	0.22 ± 0.020	1.46 ± 0.136	15.7 ± 0.5
2	Saline	37 ± 5.0	509 ± 36	0.29 ± 0.009	1.48 ± 0.926	14.3 ± 1.1
2	"ICSH"	41 ± 1.1	552 ± 28	0.27 ± 0.008	1.50 ± 0.060	17.3 ± 0.4
3	Saline	41 ± 2.1	486 ± 23	0.30 ± 0.008	1.42 ± 0.048	15.2 ± 0.6
3	"ICSH"*	40 ± 2.5	572 ± 24	0.27 ± 0.006	1.52 ± 0.050	28.6 ± 3.0
4	Saline	43 ± 3.0	549 ± 34	0.27 ± 0.019	1.47 ± 0.050	14.8 ± 0.9
4	"ICSH"*	47 ± 1.4	690 ± 42	0.23 ± 0.004	1.55 ± 0.073	43.5 ± 3.4
5	Saline	47 ± 3.4	565 ± 75	0.26 ± 0.014	1.41 ± 0.126	13.7 ± 0.4
5	"ICSH"*	52 ± 1.9	855 ± 41	0.23 ± 0.010	1.97 ± 0.044	69.8 ± 4.9
6	Saline	49 ± 3.4	574 ± 58	0.24 ± 0.007	1.36 ± 0.100	12.8 ± 0.4
6	"ICSH"	49 ± 1.9	874 ± 22	0.21 ± 0.005	1.84 ± 0.058	94.3 ± 3.4

Table 2. It is apparent that the same "dilution" effect obtained at 24 hours in the first experiment was also seen here. Beginning at about the fourth day when there was a marked increase in testis weight and in evidence of increased androgen output, as indicated by seminal vesicle weights, both free and total cholesterol expressed as

milligrams per 100 grams of rat showed a marked increase. There was apparent a trend toward slightly lower cholesterol concentrations expressed as per cent of wet weight of testis in the "ICSH" group but statistical testing of paired points revealed no significant differences.

Since it had been stated by Pollock (1942) that histo-chemically all of the cholesterol in the testis is in the interstitial tissue and since it had been asserted that there is little effect of predominantly FSH preparations demonstrable histologically on interstitial tissue (Greep, Fevold and Hisaw, 1936), it was of interest to determine whether or not the same kind of increase in testis cholesterol could be demonstrated as a result of administration of a predominantly FSH prep-

TABLE 3. EFFECT OF "FSH" ON TESTIS CHOLESTEROL CONTENT AND SEMINAL VESICLE WEIGHTS. MEANS AND STANDARD ERRORS FOR GROUPS OF 6 RATS ARE GIVEN. THE DOSE OF GONADOPHYSIN ("FSH") WAS 10 U. TWICE DAILY, SUBCUTANEOUSLY

Days of injections	Treatment	Body wt.	Testis Wt. mg./100 gm. of rat	Total cholesterol p.c. testis (wet wt.)	Total cholesterol mg./100 gm. of rat	Ester cholesterol mg./100 gm. of rat	Seminal vesicle wt. mg./100 gm. of rat
1	Saline	39±3.1	491±52	0.30±0.018	1.43±0.090	0.20±0.033	14.1±1.47
1	"FSH"	40±2.0	747±41	0.21±0.007	1.55±0.094	0.39±0.037	14.6±1.61
6	Saline	52±2.0	651±42	0.24±0.004	1.55±0.060	0.10±0.013	12.9±0.84
6	"FSH"	51±3.6	1038±49	0.25±0.005	2.57±0.005	0.46±0.030	22.8±1.62

aration as had been shown to follow the injection of "ICSH" in the preceding experiment. Therefore, two groups of animals were given 10 units of "FSH" twice daily. One group was killed 24 hours after the first injection and the other group six days after the first injection. When both of these groups of animals were compared with appropriate saline-injected controls (Table 3), it was found that the 24 hour "FSH" animals exhibited the same decrease of cholesterol expressed as per cent of wet weight of testis as did the "ICSH" animals. The six day "FSH" group showed an even greater increase in both total and free cholesterol than did the "ICSH" animals treated for a comparable length of time. It was also apparent from a consideration of seminal vesicle weights that the "FSH" animals had a comparatively small augmentation of androgen output in this experiment. On further examination of these data it was found that the absolute quantity of esterified cholesterol per 100 grams of rat was significantly increased above that of the saline controls in both the 24 hour and the 6 day groups.

Since the critical change in the adrenal cholesterol following the administration of adrenocorticotrophic hormone was confined to the esterified cholesterol fraction (Sayers et al., 1946), and since a particularly striking increase in testis cholesterol ester was noted in the 6 day "FSH" group, whereas no such increase was seen in the 6 day "ICSH" group, an experiment was designed to focus attention on the fluctuations in the ester fraction specifically. Groups of 6 animals

received "ICSH," "FSH," and saline, as summarized in Table 4. Treatment was begun on the twenty-second day of life. When the animals were sacrificed, testes and seminal vesicle weights were obtained and the testes analyzed for free and total cholesterol as before. It will be seen (Table 4) that there is no statistically significant difference between the esterified cholesterol expressed as milligrams per 100 grams of rat of the "ICSH" groups (C, D) and the saline injected controls (A, B). However, in the 6 and 10 day "FSH" groups (E, F) there is a striking increase in this fraction. Moreover, in Group H,

TABLE 4. EFFECT OF PRE-TREATMENT WITH "FSH" ON TESTIS CHOLESTEROL RESPONSE TO "ICSH," AND ON SEMINAL VESICLE WEIGHT. DOSES OF "FSH" AND "ICSH" AS IN TABLE 2. (*SIGNIFIES MEAN AND STANDARD ERRORS OF A GROUP OF 5 RATS. ALL OTHER GROUPS CONTAINED 6 ANIMALS)

Group	Days Treated	Treatment	Final Body wt.	Testis wt. mg./100 gm. of rat	Total cholesterol p.c. testis (wet wt.)	Total cholesterol mg./100 gm. of rat	Ester cholesterol mg./100 gm. of rat	Seminal vesicle wt. mg./100 gm. of rat
A	6	Saline	52±1.9	598±13	0.23±0.009	1.33±0.065	0.11±0.024	16.9± 1.0
B	10	Saline	68±1.8	912±74	0.19±0.007	1.82±0.110	0.10±0.014	18.0± 2.5
C	6	"ISCH"	46±4.7	965±34	0.20±0.004	1.96±0.031	0.13±0.018	104.0± 5.3
D	10	"ISCH"	61±6.0	1233±42	0.19±9.004	2.33±0.036	0.15±0.024	196.0±16.0
E	6	"FSH"	53±3.3	1183±33	0.23±0.008	2.67±0.050	0.42±0.049	32.1± 4.2
F	10	"FSH"	70±4.4	1288±63	0.22±0.004	2.89±0.142	0.60±0.037	38.6± 3.0
G	6 4	Saline "ISCH"	65±4.8	890±51	0.20±0.007	1.78±0.117	0.14±0.044	57.0± 4.7
H	6 4	"FSH" "ISCH"	65±3.2	1112±16	0.19±0.003	2.17±0.046	0.14±0.025	113.0±13.2
I	10	Saline	59±3.9	734±45	0.20±0.016	1.46±0.069	0.09±0.008	15.0± 1.1
J	6	"FSH"	46±2.3	1245±28	0.19±0.014	2.24±0.134	0.32±0.019	47.2± 1.4
K*	6 4	"FSH" Saline	55±1.9	876±16	0.25±0.031	2.07±0.143	0.34±0.017	23.0± 1.7

which had been treated with "FSH" for 6 days and subsequently treated with "ICSH" for 4 days the ester cholesterol returned to approximately the same level as that of the controls. Comparing the seminal vesicle weights of this group with those of another "ICSH" group (G) which had not been primed with "FSH," the synergism between these two preparations in their effect on androgen output is evident (see Greep and Van Dyke, 1942).

In order to prove that the increased androgen output of the "FSH" primed animals was associated with a true fall in the cholesterol ester fraction it was necessary to show the effect of treating with "FSH" for 6 days and subsequently treating with saline for 4 days. This experiment was done on another group of animals with a different lot of "FSH." The results, shown in Table 4, (see groups I, J, and K) indicate that in spite of the marked regression in testis size in 4 days, the cholesterol ester presisted at a high level in group K after the withdrawal of "FSH" treatment. The seminal vesicles, like the testes, showed a marked reduction in size 4 days after the treatment was discontinued.

DISCUSSION

While there are similarities between the gonads and the adrenals, certain chemical differences were apparent in the first experiment. The cholesterol content of the normal adrenals may be as high as 6 per cent of the wet weight of the glands, whereas in the 21 day old rat the corresponding figure for the testis is approximately 0.3 per

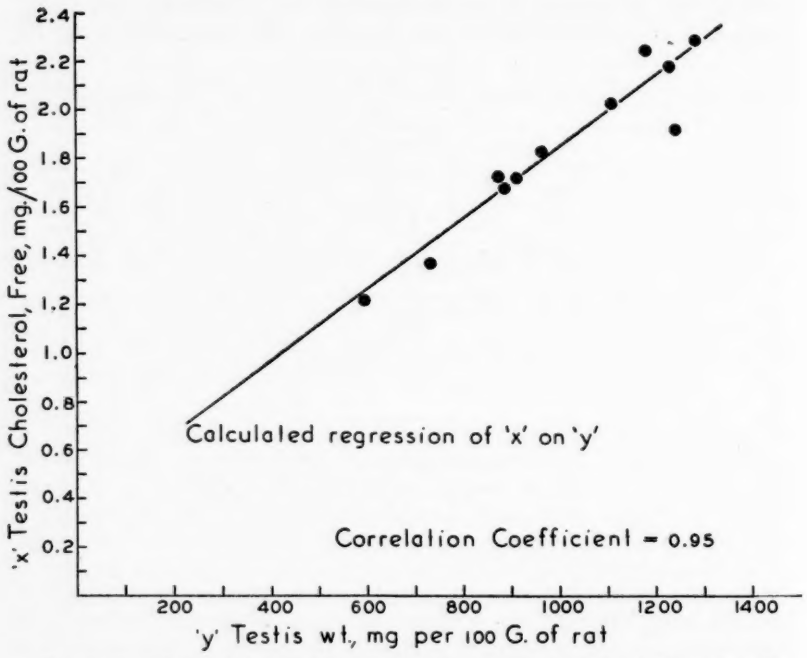

FIG. 1. Correlation between the free cholesterol content of the testis and testis weight in 11 groups of animals treated with saline, "ICSH," "FSH" and various combinations of these. Plotted from data given in Table 4.

cent. Most of the adrenal cholesterol is in the esterified form, whereas only about 10–15 per cent of the testis cholesterol of the weanling rat is esterified.

A comparison of the free cholesterol expressed as mg. per 100 gm. of rat with the testis weight in mg. per 100 gm. of rat reveals a high degree of correlation without regard to whether rats in the different groups were treated with "ISCH," "FSH," or saline (Figure 1, plotted from data in Table 4). On the other hand, the ester cholesterol in mg. per 100 gm. of rat exhibits a striking constancy over the entire testis weight range for saline-injected, "ICSH"-injected and "FSH"-primed, "ICSH"-injected rats, whereas the means of the groups that received only "FSH" all deviate significantly from those of any of the

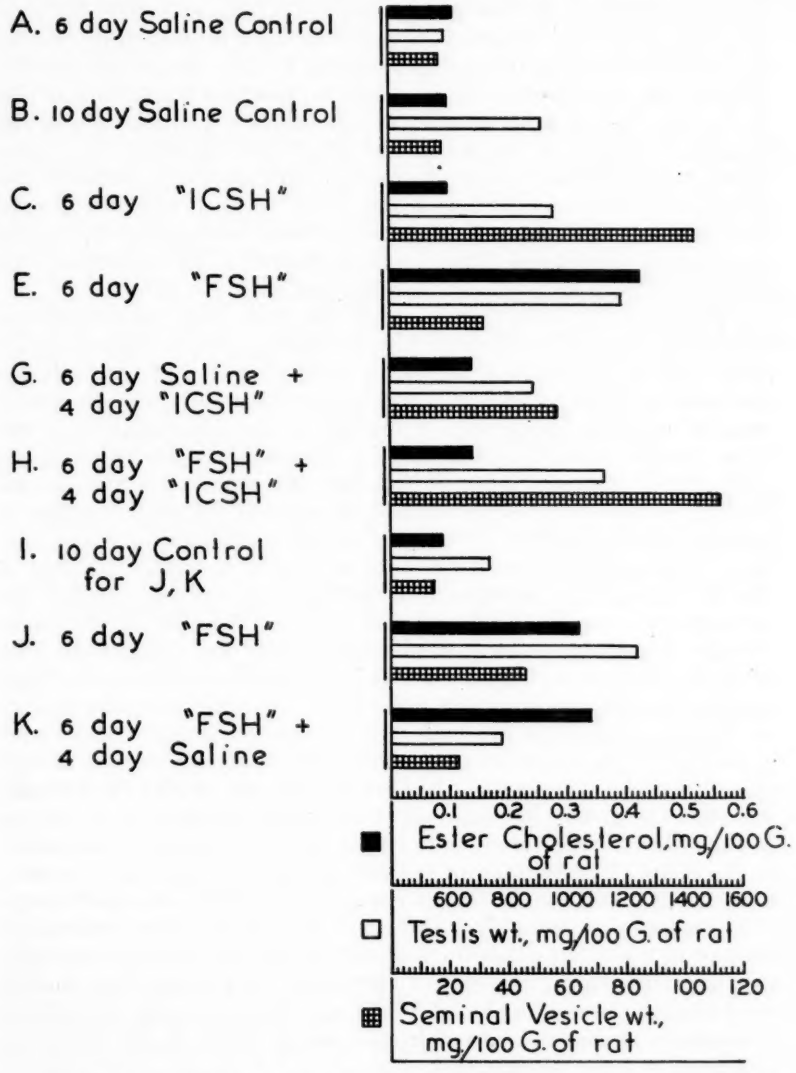
A. 6 day Saline Control
B. 10 day Saline Control
C. 6 day "ICSH"
E. 6 day "FSH"
G. 6 day Saline + 4 day "ICSH"
H. 6 day "FSH" + 4 day "ICSH"
I. 10 day Control for J, K
J. 6 day "FSH"
K. 6 day "FSH" + 4 day Saline
0.1 0.2 0.3 0.4 0.5 0.6
Ester Cholesterol, mg/100 G. of rat
600 800 1000 1200 1400 1600
Testis wt., mg/100 G. of rat
20 40 60 80 100 120
Seminal Vesicle wt., mg/100 G. of rat

FIG. 2. Fluctuations in the ester cholesterol content of the testis, testis weight, and seminal vesicle weight in animals treated with gonadotrophins. Capital letters correspond to designation of these groups in Table 4, which contains standard errors of the means given here.

others (see Figure 2, plotted from Table 4). This analysis strongly suggests that the changes in the absolute quantities of free testis cholesterol reported here may be only secondarily related to the

administration of the two gonadotrophic substances, while the fluctuations in the ester fraction appear to be more immediately affected by administration of the gonadotrophins. In the case of the "ICSH" preparation it is apparently necessary to produce a preliminary rise in the ester fraction before one can demonstrate a diminution in the esterified cholesterol content of the gland (see Figure 2). Since this substance produced marked increases in seminal vesicle weight in the un-primed animals, as well as in the "FSH"-primed ones, it can only be inferred that it might be possible to detect changes in the rate of turnover of the esterified fraction of cholesterol after the administration of ICSH by means of isotopically labelled cholesterol.

The two preparations examined in this study were selected for use because it was believed that they exhibited marked differences in their ability to stimulate androgen production and in their follicle-stimulating activity. It is of some interest that they may be differentiated also by means of a quantitative chemical analysis of the male gonad. Gonadophysin ("FSH") produces an increase in ester cholesterol (expressed both as mg. per 100 gm. of rat and as per cent of total testis cholesterol) and comparatively small changes in seminal vesicle weight. Follutein ("ICSH") produces a fall in the ester cholesterol of the testis only in "FSH"-primed rats, but a marked increase in androgen production in un-primed rats and an even greater one in "FSH"-primed animals. Whether or not these changes are of physiologic significance cannot be stated until this work is repeated with purified FSH and ICSH preparations of hypophysial origin, since, clearly, one cannot attribute such significance to changes obtained in the male with a chorionic gonadotrophin and a mixture of sheep pituitary gonadotrophins. In any case it is suggested that the changes described here are consistent with the hypothesis that the gonadotrophins affect androgen production by virtue of their direct involvement in the metabolism of esterified cholesterol in the testis. A description of the precise nature of this involvement must await further work. One can only suggest that some constituent of Gonadophysin, possibly FSH, may increase the ester cholesterol content of the gland either by accelerating the rate of local esterification or by increasing the rate of abstraction of pre-esterified cholesterol from the circulating blood. ICSH may function in the conversion of esterified cholesterol into androgen and, possibly, in the release of androgen into the bloodstream.

SUMMARY

1. A single subcutaneous dose of a potent interstitial cell stimulating chorionic gonadotrophin (Follutein, Squibb) resulted in an apparent fall in per cent of both free and total cholesterol in the testis of the immature rat at 24 hours after injection. This was shown to be due to hydration of the glands.

2. Immature rats injected twice daily for 6 days with 20 U. of "ICSH" showed testicular hypertrophy, marked increases in seminal vesicle weight and increases in both free and total cholesterol expressed as mg. per 100 gm. of rat. Cholesterol ester (mg. per 100 gm. of rat) was not significantly different from that of control rats.

3. The administration of a predominantly follicle-stimulating concentrate of sheep pituitary glands (Gonadophysin, Searle) produced significant increases in testis cholesterol ester (mg. per 100-gm. of rat) after 1 and 6 days of treatment. There was comparatively little increase in seminal vesicle weight in these animals.

4. Four-day treatment with "ICSH" of animals that had previously been treated with "FSH" for 6 days resulted in a striking fall in testis cholesterol ester associated with evidence of markedly augmented androgen production.

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NOTES AND COMMENTS

VAGINAL CORNIFICATION IN SPAYED RATS BY INTRAVAGINAL APPLICATION OF NON-ESTROGENIC SUBSTANCES*

IN THE course of investigations of the estrogenic activity of human and rabbit blood (Krichesky and Glass, 1947) it became apparent that vaginal cornification may be induced by substances other than estrogens. Bioassay was carried out in the spayed rat according to the method described by us. Positive responses elicited in this fashion were obtained not only with blood from normal rabbits but also with blood from spayed rabbits; an average value of 308 I.U. per 100 cc. whole blood was found in the former whereas 150 I.U. per 100 cc. was obtained from the latter. Estrogenic activity was also observed in the blood from castrated human females. This latter finding is not in agreement with that of Albrieux (1941) who introduced the method of estrogen bioassay by intravaginal pellets and who reported absence of vaginal cornification following the use of pellets of whole dried blood from a surgically castrated woman.

The responses to the blood of both normal and castrated individuals stimulated our interest in the mechanism of vaginal cornification by non-estrogens.

That minor trauma incident to frequent swabbing *per se* will induce vaginal cornification has been demonstrated repeatedly. There was also the question of a foreign body reaction set up by the vaginal pellets. It was considered possible that this may have been in the nature of a chemical irritation or possibly that of a metabolic interplay between the blood proteins and vaginal mucosa. The possibility existed also that extragonadal substances may have been present in blood from castrated women which were capable of inducing vaginal cornification. If, on the other hand, the factor of an artefact were shown to exist, then it should be possible to arrive at the true estrogenic activity of blood by subtracting the values obtained in blood after castration from that observed in non-castrated individuals.

MATERIALS AND METHODS

The test animals used were spayed female rats of the Wistar strain ranging in age from 3 to 10 months. These were standardized by the method described by us (Krichesky and Glass, 1947) so that 0.1 i.u. of crystalline estrone in sesame oil produced a positive response in 50 per cent of the animals.

The dry materials used were powdered in a mortar with pestle and were hammered into pellets of approximately 2 mm. in diameter. Ten per cent sesame oil was added to act as a binder. The pellets were cut into desired

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lengths and weighed. Each pellet was then cut into two approximately equal parts and each portion introduced intravaginally into an animal 24 hours apart to simulate divided doses used in estrogen assays. As control material, small pellets of high melting point paraffin (62 to 68 degrees C.) and glass beads were used. These were made to approximate the same length and diameter of the blood pellets.

Liquid materials used were instilled intravaginally in two doses of 0.05 ml. each 24 hours apart, employing a blunt No. 18 needle attached to a one cc. syringe.

RESULTS

All pertinent data, including the materials used, dosage, number of animals and their response are contained in Table 1. The data indicate that

TABLE 1. CORNIFICATION OF THE OVARIECTOMIZED RAT'S VAGINA AFTER INTRAVAGINAL INTRODUCTION OF NON-ESTROGENS

Compound	Size of pellet	No. of animals	% positive
Paraffin	2–3 mm. diam.	16	6
Glass beads	2–3 mm. diam.	16	0
Sesame oil	0.1 cc.	20	0
Egg white	10 mg.	10	40
	20 mg.	11	55
	40 mg.	11	73
Gelatin	5 mg.	10	100
	20 mg.	10	100
Cystine	5 mg.	6	66
	10 mg.	10	70
	20 mg.	4	75
Cholesterol	10 mg.	10	70
	20 mg.	8	75
	40 mg.	8	88
Corn starch	8 mg.	12	17
	30 mg.	12	66
Benzene / Sesame oil (equal parts)	0.1 cc.	10	90
Benzene (1 part) / Sesame oil (2 parts)	0.1 cc.	10	80
Control	—	20	0

all of the substances employed except paraffin, glass beads and sesame oil induce vaginal cornification when introduced intravaginally into spayed female rats. Only one of 16 animals receiving paraffin pellets showed cornification and this was, in all probability, due to mechanical trauma. The great variety of chemical substances represented by the compounds used suggests that the positive responses obtained bear no relation to the chemical structure. Nor is it likely that these compounds stimulate growth of the vaginal epithelium through a possible nutritive or metabolic effect, since benzene also produced cornification. The most reasonable explanation that suggests

itself is that the cornification induced by these compounds is merely a chemical irritation, either directly as in the case of benzene, or indirectly by bacterial growth and subsequent irritation by metabolites. The fact that in all instances in which cornification was seen (except in the case of benzene) a rich bacterial flora and leukocytic infiltration were observed lends support to this hypothesis.

SUMMARY AND CONCLUSIONS

These observations demonstrate that a variety of non-estrogens introduced directly into the vagina of spayed female rats may induce cornification of the vaginal mucosa and that such effects are probably due to chemical irritation.

In spite of our demonstration that non-estrogens may induce vaginal cornification in spayed rats, the intravaginal introduction of whole dried blood reported in a previous publication (Krichesky and Glass, 1947) may still be useful in approximating levels of circulating estrogens. In a series of seven castrated female rabbits and four surgically castrated women (some of these data unpublished) it was found that calculated estrone equivalents in these bloods ranged from 120 to 210 I.U. per 100 cc. whole blood, while values from the blood of non-castrated women were always higher.

The cornification produced by the blood from castrated women may be due to chemical irritation of non-estrogens and/or to estrogens of extragonadal origin and varies within a limited range. The higher values obtained with blood from intact animals may well be due to circulating estrogens. If this is true, then the difference between the average values of the bloods from castrated and non-castrated women may represent the approximate level of circulating blood estrogens.

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A FLUOROMETRIC METHOD FOR THE DETERMINATION OF ESTROGENS

THE USUAL colorimetric methods employed for the estimation of estrogens are not of sufficient sensitivity to determine the minute quantities found in normal urine (Kober, 1931; David, 1934; Zimmerman, 1935; Talbot et al. 1940; Szego and Samuels, 1943). Fluorometric analysis would appear to offer a solution to this problem due to its greater sensitivity. Finklestein et al. (1947) have recently reported in a preliminary note that when phosphoric acid is added to crystalline estrone, estradiol and estriol a fluorescent

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substance is obtained. However, the sensitivity of this method does not appear great enough for its general application and furthermore it has not as yet been adapted to biological fluids. Marrian (1930) showed that when concentrated sulfuric acid is added to estrone, an orange color with greenish fluorescence is produced. The purpose of the present report is to make available data to show the applicability of this fluorescent phenomenon to the quantitative determination of estrogens in urine.

METHODS AND MATERIALS

In the analytical method employed, organic reagents are re-distilled and all-glass apparatus is used throughout in order to prevent the appearance of extraneous fluorescence. Dow Corning stop-cock grease is employed. In order to attain maximum fluorescence without charring, 60% sulfuric acid (Mallinckrodt, low-nitrogen) is used. In the procedure 8.0 ml. of 60% H_2SO_4 are added to 0.5–1.0 ml. methyl alcohol (containing 0–0.25 micrograms of estrone or estradiol) and heated in a boiling water bath for 5 minutes and then cooled. Fluorescence is read in a Coleman Photofluorometer Model #12 using a B-2; PC-9A filter combination. A linear relationship has been established between the concentration of both estrone and estradiol and the galvonometer reading, as shown in Table 1.

Table 1. Linear Relationship Between Concentration of Estrogen and Galvanomfter Readings in Coleman Photofluorometer

		Filter B-2; PC-9A	Filter B-1; PC-9A
Estrone	0.1 microgram	40	6
	0.15 microgram	61	7
	0.2 microgram	80	7
Estradiol	0.1 microgram	40	4
	0.15 microgram	58	6
	0.2 microgram	81	7
Estriol	0.5 microgram	12	2
	1.0 microgram	24	4
	2.0 microgram	42	5

The above procedure has been employed to study the fluorescence of other steroids. Estriol yields about 1/20th the fluorescence found with estrone and estradiol. Testosterone, progesterone, desoxycorticosterone, and cholesterol also fluoresce but to an even lesser degree. For example, the fluorescence of cholesterol is approximately 1/200th that of estrone and estradiol. Androsterone, dehydroandrosterone and pregnandiol do not fluoresce at the concentrations of 20 μg. Equilin and equilinin have not been tried.

In order to prepare biological material for the above analytical method, the extraction technique of Bachman and Petit (1941) was modified in certain details. The hydrolyzed urine was extracted directly with benzene and the acid washes have been eliminated. For the fluorometric determination of estrogens in normal male and female urines, only a 10 ml. aliquot is necessary. For pregnancy urines obviously much smaller amounts are used. It is

essential that in all analyses, reagent and absolute blanks are made for each determination.

In order to establish valid results, the fluorescence in the biological extracts is read at two different wave-lengths, 4360 A (B-2 filter) and 3650 Å (B-1 filter). The non-specific fluorescence of the reagent blanks and other non-estrogenic substances are similar at the two wave-lengths while that of estrone and estradiol are quite specific at 4360 A. Thus, by reading the solutions with the two filters the blank fluorescence may be deducted.

Recovery experiments have been performed with known amounts of estrone and estradiol added to water and urine. The percentage recovery is constant ranging between 50–60%. Several examples are presented in Table 2. This was to be anticipated owing to the distribution coefficients of the estrogens between the aqueous and organic solvents used. The addition of

TABLE 2. RECOVERY OF KNOWN AMOUNTS OF ESTROGENS WHEN ADDED TO EITHER WATER OR URINE AND EXTRACTED AS DESCRIBED

Estrogen added	Micro-grams	Substance	Preformed estrogen	Total estrogen	Estrogen recovered	% recovery
Estrone	0.2 μg.	water	0	0.104	0.104	52%
Estrone	0.2 μg.	urine	0.11	0.22	0.11	55%
Estradiol	0.2 μg.	water	0	0.12	0.12	54%
Estradiol	0.2 μg.	urine	0.27	0.38	0.11	55%

cholesterol, progesterone, pregnandiol, androsterone and dehydroandrosterone to urine does not interfere with either estrogen determinations or with recovery experiments.

RESULTS

A number of observations were made in order to establish that the material being estimated in the various normal and pathological urines is in reality estrogenic material. Evidence derived is four-fold in nature:

1. Urine of 2 normal young women has been studied through the menstrual cycle by daily analysis for estrogen. The results obtained are in complete agreement with those reported in the literature with biological assay, i.e., there is a peak excretion at mid-interval and another preceding menses.

2. The concentration of estrogen in late pregnancy and stallion urine has been found to be high (1.5 mg./liter and 12.24 mg./liter respectively), the concentration has been found to be low in males (21 μg/day), women with amenorrhea (15.2 μg/day) and in the post-menopausal phase (2.9 μg/day).

3. Administration of 2 different estrogens to two normal young men resulted in a marked rise in total daily excretion. For example, in one individual the excretion rose from 21.6 μg. to a level of 116 μg.

4. Finally, satisfactory correlation between the biological assay and the fluorometric method has been obtained on urines extracted in identical fashion.

DISCUSSION

The fluorometric method presented above has a very high order of sensitivity and consequently can be used to estimate the minute amounts found in as little as 10 ml. of normal urine. Interfering substances which fluoresce are largely excluded in the determination by differential solubility. It has

been shown that there is a general correlation between biological evidence of estrogenic activity and the fluorometric method described. Detailed description of the results will form the basis of another communication.

SUMMARY

A fluorometric method for the estimation of estrone and estradiol is described. This method appears to have value in the specific determination of estrogens in urine.

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THE DIABETOGENIC ACTIVITY OF ALLOXAN IN OLD AND YOUNG RATS

IN A recent paper by Lazarow and Palay (1946), figures are reported for the incidence of diabetes in rats after intravenous injection of alloxan in various doses. The Effective Dose 50 (ED 50) was not calculated by the authors, but it is evident that their rats became diabetic with much lower doses than those used in our laboratory (Brückmann and Wertheimer, 1947). Although strain differences in the susceptibility of rats to alloxan are known to occur (Goldner and Gomori, 1943), another plausible explanation might be suggested, namely that Lazarow and Palay used adult rats with an average weight of about 200 gm., while our rats were 2–3 months old, weighing about 100 gm.

In order to decide this point, a group of 7–13 months old rats, with an average weight of 250 gm., was studied. Simultaneously, an additional group of young rats (average weight 100 gm.) was investigated, to avoid errors due to possible seasonal variations in the sensitivity of rats to alloxan. These two groups, which were observed during December 1946–April 1947, are referred to hitherto as old and young rats, respectively. Our experimental technique was practically identical with that of Lazarow and Palay, and it has been described in detail in our report. As an indication of the presence of diabetes these authors took a blood sugar level exceeding 160 mg. per 100 cc., while we have taken the appearance of glucosuria during an observation period of at least 5 days as sign of diabetes.

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The ED 50, b, the slope of the regression line, and their respective Standard Errors have been calculated according to Litchfield and Fertig (1941) and Miller and Tainter (1944). Both methods are graphical simplifications of the fundamental treatment of the dose-effect relationship introduced by Gaddum and by Bliss.

Although it was observed during the experiments that the old rats developed more severe diabetes than the young animals treated with equal doses, this difference is not apparent in the ED 50 (uncorrected) values shown in Table 1. Obviously, the all-or-none response type of assay applied here only gives a satisfactory measure of the activity of a drug, if the degree of the response itself does not vary too widely. In our case, the simple recording of presence or absence of glucosuria ("diabetes") does not take note sufficiently of the great variations in the degrees of diabetes actually observed. This can be corrected by the introduction of a scoring technique,

TABLE 1. THE ED 50 (MG. PER KG.) AND SLOPE OF REGRESSION LINE IN OLD AND YOUNG RATS TREATED WITH ALLOXAN

	ED 50 ± S.E.		Slope (b) ± S.E.		N		N′	
	old	young	old	young	old	young	old	young
Uncorrected	39.0±1.13	38.0±1.58	15.9±2.9	12.0±2.3	67	95	50	52
Corrected	41.4±1.17	48.3±2.33	16.4±3.1	9.1±1.0	67	79	50	56

N = Total number of rats; N′ = Number of rats within probibits 3.5–6.5.

and the following simple and objective system was applied: Severely diabetic rats (with ketonuria) were given the score 3, lightly diabetic rats (late or transient diabetes, see Brückmann and Wertheimer, 1947) score 1, and all other diabetics in between, score 2. To avoid percentages exceeding 100, the sum of the scores for each dose was divided by 3, and the resulting figure used as usual for calculation of the percentage of diabetics at each dose.[1]

The ED 50 (corrected) values thus obtained are presented in Table 1. This ED 50 is essentially the dose of alloxan causing diabetes of a certain, defined degree in 50% of the animals. The difference between young and old rats, about 17% is highly significant ($p = 0.01$). The greater slope of the regression line in the case of the old group means that the response to alloxan was here more uniform; whether this is characteristic for old rats cannot yet be stated. The ED 50 (uncorrected) calculated from Lazarow and Palay's figures is 26 mg. per kg. Although it is not strictly comparable with our value, it is clear that it is much lower than ours, so that a real difference between their rats and ours of the same age exists, and is probably due to a difference in strains.

Another method to demonstrate the higher sensitivity of old rats to alloxan is used in Table 2. Here the blood sugar response and incidence of ketonuria are compared at equal doses. In contrast to Lazarow and Palay (1946), we use fasting blood sugar values, which we have found to be more uniform than the non-fasting ones. The observed figures in old rats receiving 40 mg. per kg. of alloxan correspond roughly to the values which we have obtained in young rats receiving 50–60 mg. per kg.

The differences between old and young rats shown in Table 2 are sta-

[1] This procedure will be discussed more in detail in a later report dealing with observations on several hundred alloxan-treated young rats.

tistically significant. For the blood sugar values, $p = 0.05$ (small sample method); for ketonuria, $p = 0.02$ (Chi square test, Yates' modification).

These findings can possibly be explained in the following way. The degree of pancreatic damage caused by equal doses of alloxan is not very different in old and young rats. Old rats, however, are perhaps more sensitive towards the resulting insulin-deficiency and consequently develop more severe diabetes. In addition, repair of the damaged tissue can be assumed to be more active in young rats, and contributes thus towards the alleviation of existing diabetes. The same conclusion has been recently reached by Martinez (1947) in Houssay's laboratory, based on work with partially pancreatectomized old and young rats. Martinez (1947) mentions that the pancreas of young

TABLE 2. FASTING BLOOD SUGAR (3 DAY) AND KETONURIA IN OLD AND YOUNG RATS AFTER 40 MG. PER KG. ALLOXAN. DIABETIC RATS ONLY

Group	Mean blood sugar ± S.E.	Incidence of ketonuria
Young	142 ± 11.4 (9)	0/14
Old	280 ± 56 (13)	6/17

Number of animals in brackets.

rats contains a relatively larger number of islet cells than the adult pancreas. If this is true, another and simpler explanation for our findings can be offered. The "true" alloxan dosage, namely mg. drug per kg. of β-cells, would be in this case higher in old than in young rats, and consequently the resulting damage to these cells more severe.

SUMMARY

1. The Effective Dose 50 and the slope of the regression line have been determined in old and young rats treated with alloxan. The values are nearly identical when no corrections are applied for the severity of the resulting diabetes, but with appropriate corrections the ED 50 is significantly lower in old rats.

2. With equal doses, much higher blood sugar values and a higher incidence of ketonuria are observed in old diabetic rats than in young animals.

3. In all-or-none response assays, in which there are wide variations in the degree of the response, it is desirable to introduce correction factors for these variations, which enable a better definition of the degree of the response.

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HYALURONIDASE ACTIVITY OF SPERMATOZOA

THE enzyme, hyaluronidase, which has been found, among other sources, in mammalian semen has been implicated in the process of fertilization by the demonstration that it readily disperses the cumulus cells of mammalian ova (McClean and Rowlands 1942; Fekete and Duran-Reynals, 1943; Leonard and Kurzrock, 1945). This finding has led to the view that the millions of spermatozoa required to induce fertilization of a few ova, are necessary because they furnish hyaluronidase in sufficient concentration to remove the tightly adhering cumulus cells from the ova and thus permit sperm penetration (Rowlands, 1944).

In considering the role of hyaluronidase in fertilization, it has been tacitly assumed that (a) spermatozoa contain hyaluronidase and (b) this spermatic hyaluronidase is readily diffusible, despite the fact that there is no direct evidence to support either assumption. The indirect evidence in agreement with the view that the hyaluronidase content of semen is due to spermatozoa, could also be satisfactorily accounted for by an alternative hypothesis; namely, that the hyaluronidase in semen is derived from testis but not from spermatozoa, that the enzyme is present in the seminal plasma and that its concentration in semen is controlled by factors which also influence spermatogenesis. In order to distinguish between these possibilities, studies on the hyaluronidase activity of semen were undertaken.

Rabbit semen (collected with an artificial vagina) was utilized in these experiments. In all instances the semen was studied within 24 hours (during which time the sample was refrigerated) and in most cases within one hour after obtaining the sample from the rabbit. Hyaluronidase activity was measured using either a viscometric (Hadidian and Pirie, 1947) or a skin spreading method of assay (Hechter, 1947). The latter method, while sensitive and rapid, is useful for semiquantitative work where large differences are expected; the former method is quantitative but is less sensitive.

In the first experiments, the hyaluronidase content of whole semen was compared to that of semen which was centrifuged to remove the spermatozoa (seminal plasma). Under the conditions of centrifugation employed (15 minutes at a speed of approximately 2000 RPM) about 2 per cent of the spermatozoa are not sedimented and accordingly the seminal plasma samples obtained are not completely sperm-free. Using the viscometric method of assay on undiluted semen, it was observed that the seminal plasma contained approximately half as much activity as the same semen containing spermatozoa. Studies on the hyaluronidase content of semen from vasectomized rabbits revealed a complete absence of hyaluronidase using both methods of assay.

These findings demonstrate that the hyaluronidase activity in semen is derived from a portion of the generative tract above the vas deferens and that a portion of the enzyme is associated with the spermatozoa. However, these results do not provide information concerning the question as to whether the hyaluronidase in the seminal plasma is derived from the spermatozoa, or whether the source of the enzyme associated with the spermatozoa is the seminal plasma.

To obtain information on this point, the following procedure was em-

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ployed. A semen sample (diluted 1:100 with physiological saline when the spreading method of hyaluronidase assay was used, and undiluted when the hyaluronidase was measured in the viscometer) was centrifuged to yield a clear seminal plasma and a pellet containing the bulk of the sperm. The seminal plasma was removed and the hyaluronidase activity of this sample was measured. The pellet in the centrifuge tube was resuspended in an amount of saline equivalent to the original volume of semen, allowed to stand for periods of time ranging from 2 to 10 minutes at room temperature and then recentrifuged to yield another clear fluid. This fluid represents a second "washing" of sperms, and a sample of this material was tested for hyaluronidase activity. In similar fashion the third and in some cases the fourth to seventh washings of spermatozoa were obtained and assayed for hyaluronidase activity. The results of this type of experiment may be summarized briefly as follows: (a) Using the spreading method of assay it was consistently observed that the hyaluronidase concentration in the 2nd, 3rd, 4th, 5th, and 6th washings remained approximately equivalent to the original seminal plasma until the 7th washing, at which time the enzyme activity fell off at least 10 fold. (b) Using the viscometric method of assay, it was observed that the 2nd and 3rd washings contain as much hyaluronidase as did the original plasma; no viscometric measurements of enzyme activity were made on further washings. These findings indicate that the hyaluronidase activity obtained in the washings of sperm must be derived either from the spermatozoa or is closely associated with them. In addition, these results demonstrate that the hyaluronidase associated with spermatozoa is readily diffusible into the medium since the "washed" spermatozoa were suspended in saline for at most 10 minutes and in the case of the viscometric studies, the suspension period was of the order of 1 to 2 minutes. Finally, it should be mentioned that these results indicate that normal rabbit spermatozoa either contain, or produce under our *in vitro* conditions, a large amount of hyaluronidase relative to the amount of enzyme which diffuses into the original seminal plasma.

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ASSOCIATION NOTICE

THE E. R. SQUIBB AND SONS AWARD

The E. R. Squibb & Sons Award of $1,000.00 was established in 1939, and was given first in 1940 to Dr. George W. Corner; 1941—Dr. Philip E. Smith; 1942—Dr. Fred C. Koch; 1943—no award was given; 1944—Dr. E. A. Doisy; 1945—Dr. E. C. Kendall; 1946—Dr. Carl G. Hartman; 1947—Drs. Carl F. and Gerty T. Cori. A special Committee of five members of the Association chooses an investigator or investigators in the United States or Canada for one of the best contributions to endocrinology.

THE CIBA AWARD

The Ciba Award to recognize the meritorious accomplishment of an investigator not more than 35 years of age in the field of endocrinology was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the award was presented to Dr. E. B. Astwood; 1945—Dr. Jane A. Russel; 1946—Dr. Martin M. Hoffman; 1947—Dr. Choh Hao Li. The work cited may be either in the field of pre-clinical or clinical endocrinology. The Award is for $1,200.00. If within 24 months of the date of the award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, the Award will be increased to $1,800.00.

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The Ayerst, McKenna & Harrison Fellowship was first awarded in 1947 to Dr. Samuel Dvoskin. The fellowship was founded in order to encourage investigation in the field of endocrinology rather than as an award for work done. The amount of the fellowship is $2,500.00 annually. The nominee must possess the degree of Doctor of Philosophy or Doctor of Medicine or their equivalent. It is suggested that no restriction be placed on age, but that preference be given to applicants who have recently completed the requirements for their Ph.D. or M.D. degree. The nominee must present evidence of scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals; submit a program of proposed study; indicate one or more institutions where the proposed program shall be carried out; submit statement of approval from the investigators with whom he proposes to conduct his research; serve full time if awarded a fellowship. A small amount of time (10 to 15 per cent) may be allotted for course work or for participation in teaching, the latter purely on a voluntary basis.

Each member has the privilege of making one nomination for each Award. A nomination should be accompanied by a statement of the importance of the nominee's contributions in endocrinology and by a bibliography of the nominee's most important publications, and reprints if possible. The nominations should be made on special application forms which may be obtained from the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City 3, Oklahoma, and returned to him not later than March 15, 1948.